ISOLATION AND CHARACTERIZATION OF β-LACTAMASE AND MBL PRODUCING BACTERIAL ISOLATES FROM HOSPITAL SETTINGS

ABSTRACT
OF THE
THESIS
SUBMITTED FOR THE AWARD OF THE DEGREE OF

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IN

BIOTECHNOLOGY

By

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Under the supervision of
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Antibacterial resistance is a natural biological phenomenon of response of bacteria to the selective pressure of an antibiotic. The overuse and misuse of antibiotics in humans and in animal husbandry has been cited as a responsible factor in the development of drug resistance in all bacterial species. Emergence of multidrug resistance is more worrisome in entire medical fraternity. Multidrug-resistant strains possessing extended spectrum β-lactamases (ESBLs) has become an increasing problem worldwide. The advancing age, female gender, low socio economics status, illiteracy, hospital cross infection, the food chain trade and human migrations have contributed to increase the risk for community-acquired ESBL. CTX-M, SHV, TEM, and OXA enzymes have become the most prevalent ESBLs. The work presented in this thesis has explored the susceptibility and resistance pattern of clinical isolates of hospital setting, risk factors for acquisition of these organisms, mode of spread of resistance, prevalence of ESBL producers among bacterial isolates of clinical origin and their molecular mechanism of resistance.

The first chapter of this study was made to (i) characterize the bacterial pathogens in patients having gram negative septicemia, (ii) evaluate the bacterial resistance and (iii) explore the underlying molecular mechanisms in these strains. 70 cases of gram negative sepsis were included in this prospective, open labeled, observational study. Standard methods for isolation and identification of bacteria were used. Antimicrobial susceptibility and ESBL testing was performed by the standard disc diffusion method. PCR amplification was performed to identify $\boldsymbol{bla}_{\text{CTX-M}}$, $\boldsymbol{bla}_{\text{SHV}}$ and $\boldsymbol{bla}_{\text{TEM}}$ type ESBLs. Conjugation experiment was performed to show resistant marker transfer. The most prevalent isolates *E. coli* 58.6%, *Klebsiella* spp. 32.9% and *Pseudomonas* 8.6%, were resistant to most of the antimicrobials including cefazolin, ceftriaxone, cefuroxime,

ampicillin and co-trimoxazole but sensitive to imipenem and meropenem. ESBL and MBL production was seen 7.3% and 12.2% of *E. coli* isolates, respectively. Three isolates were found to have $bla_{CTX\text{-}M\text{-}15}$ and two of them also showed $bla_{TEM\text{-}1}$ type enzymes. Whereas, none of them was observed to possess bla_{SHV}. Conjugation experiments using J-53 cells confirmed these resistant markers as plasmid mediated. The study highlights the molecular epidemiology of escalating antimicrobial resistance and likely switch over of $bla_{CTX\text{-}M\text{-}15}$ type extended spectrum β-lactamases by bla_{TEM} type ESBLs in India. Further, the antimicrobial resistance by horizontal gene transfer was predominant among *Enterobacteraceae* in the community setting.

Second chapter of this study was designed to (i) find out the susceptibility of antimicrobial agents, (ii) identify the various risk factors associated with ESBL producing *K. pneumoniae* clinical isolates, and (iii) understand the mode of mechanism among the *K. pneumoniae* isolates circulating in neonatal intensive care unit (NICU), Aligarh hospital of North India. Clinical isolates were characterized to detect *K. pneumoniae*. Susceptibility testing was done by standard disc diffusion method. MIC was determined using agar dilution methods. ERIC-PCR and resistant marker detection was performed by PCR amplification. Plasmid analysis and transfer experiments were carried out to further type the strains. Sequence analysis of resistant marker ($bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV}) was performed. Incompatibility and multilocus sequence typing were carried out. Among the clinical isolates, 103 (37.62%) strains were identified as extended spectrum β-lactamase producing *Klebsiella pneumoniae* (EPK) while remaining 262 (71.78%) strains were non-EPK. Furthermore, 103 EPK strains were characterized according to socioeconomic status, educational status, gestational age, onset sepsis, birth weight, maternal intrapartum

symptomatic infection, prolonged rupture of membrane, infection carriage, length of stay in NICU and environmental origin. EPK were found susceptible to carbapenem, cephalosporins, gatifloxacine and amikacin. PCR amplification and sequence analysis revealed $bla_{CTX\text{-}M\text{-}3}$, $bla_{TEM\text{-}1}$ and $bla_{SHV\text{-}1}$ as predominant markers in EPK. PCR based replicon typing revealed Inc/FIC, Inc/H12, Inc/X and Inc/FIB types of plasmids. MLST analysis showed prevalence of ST-64/51/280 among the EPK carrying CTX-M-3 type ESBL on Inc FIB/H12/FIC/X types of plasmids, indicating horizontal gene transfer. Low socioeconomic status, illiteracy of mother and instrumental carriage are independent risk factors for EPK infections.

Third chapter of this study was aimed to characterize the mode of transmission of $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV}, $bla_{OXA\text{-}1}$, Arm-A and Rmt-A among extended spectrum β-lactamases (ESBL) producing *K. pneumoniae* strains isolated from NICU of a teaching hospital of North India for understanding of molecular mechanism of resistance. 3000 neonates admitted during January 2009 to February 2011 in NICU were screened for clinical isolates and characterized for *K. pneumoniae*. Susceptibility testing was done by standard disc diffusion method. MIC was determined using agar dilution methods. ERIC and replicon types were performed to type the strains. PCR amplification and sequence analysis of $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV}, $bla_{OXA\text{-}1}$, Arm-A and Rmt-A were carried out. Plasmid analysis and transfer experiments were performed to understand the mode of transfer of resistant marker. 262(33.37%) isolates were characterized in NICU as ESBL producing *K. pneumoniae* strains. These ESBL producing *K. pneumoniae* strains were found susceptible only to carbapenem, cephalosporins and amikacin. PCR amplification and sequence analysis revealed $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV}, $bla_{OXA\text{-}1}$, Arm-A and Rmt-A as

predominant markers in ESBL producing *K. pneumoniae* strains. The plasmids carrying $bla_{CTX\text{-}M\text{-}3}$ $bla_{TEM\text{-}1}$, $bla_{SHV\text{-}1}$, $bla_{OXA\text{-}1}$, Arm-A and Rmt-A genes were assigned to Inc/I1γ, Inc/FIA-FIB, Inc/FIB, Inc/HI2 and Inc/HI1 types. Large genetic background diversity in the ESBLs producing isolates and dissemination of the $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV}, $bla_{OXA\text{-}1}$, Arm-A and Rmt-A genes harboring isolates were observed within the ESBL producing *K. pneumoniae* strains of NICU of Aligarh, North India. This study revealed plasmid mediated resistance to ESBL producing *K. pneumoniae*. This study also suggests carbapenem, ceftazidime and amikacin as drugs of choice.

The fourth chapter of this study aimed at (i) identifying the MBL producing bacterial isolates in infected diabetic foot ulcerated patient, (ii) determining and quantifying the resistance and susceptibility of those isolates, and (iii) characterizing the mode of transmission of CTX-M, TEM, SHV, NDM-1, VIM, OXA and Arm-A among the MBL producing clinical isolates. Twenty clinical isolates of infected diabetic foot patients from ICU and endocrinology ward of a teaching hospital of North India were screened for MBL producing pathogens. Antimicrobial susceptibility and MBL detection were performed and reconfirmed with HiComb MIC testing strips and disk diffusion method. MIC was determined using agar dilution methods. PCR amplification and sequence analysis of $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV}, $bla_{OXA\text{-}1}$, and Arm-A were carried out. To understand the mode of transfer of resistant marker, conjugational transfer method was used. Replicon typing and MLST were performed to type the strains. Insertion sequence was also identified. Out of 20 clinical isolates of diabetic foot ulcer, 14 strains were characterized as *K. pneumoniae*, 5 were *E. coli* and one was *E. cloacae*. One *K. pneumoniae* (Kp12) and one *E. cloacae* (Ec15) were also found to be carbapenem

resistance. PCR amplification and sequence analysis revealed the presence of NDM-1 in Kp12, and Ec15 strains. ***bla***$_{CTX-15}$, ***bla***$_{SHV-1}$ in all clinical isolates, ***bla***$_{TEM-1}$ in 12 isolates (9 isolates in *K. pneumoniae*, 2 isolates in *E. coli* and one isolate in *E. clocace*) and ***bla***$_{OXA-1}$ in 7 isolates (5 isolates in *K. pneumoniae*, one isolate in *E. coli* and one isolate in *E. clocace*). Replicon typing revealed the ***bla***$_{NDM-1}$ carrying plasmid in Ec15 of incompatibility group Inc L/M. MLST analysis of Kp12 showed ST14. Genetic environment revealed remnant of IS*Aba*125 upstream of the ***bla***$_{NDM-1}$ gene in Ec15 and an entire IS*Aba*125 upstream of the ***bla***$_{NDM-1}$ gene in Kp12. Moreover, bleomycin resistance gene ***ble***$_{MBL}$ was also identified downstream of the ***bla***$_{NDM-1}$ gene in both the cases. This study reports the MBL producers having ***bla***$_{NDM-1}$ gene in *K. pneumoniae* and *E. cloacae* isolate of diabetic foot ulcer and revealed the mode of transmission and mechanism of resistance of these isolates.

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Certificate

I certify that the work embodied in the thesis entitled "Isolation and characterization of β-lactamase and MBL producing bacterial isolates from hospital settings", is an original work, unless otherwise stated, carried out by Mr. Saeedut Zafar Ali under my supervision and is suitable for submission in fulfillment of the requirements for the award of Doctor of Philosophy in Biotechnology.

Asad Ullah Khan, PhD
Associate Professor

Declaration

I, hereby, declare that the thesis entitled "Isolation and characterization of β-lactamase and MBL producing bacterial isolates from hospital settings" embodies the work carried out by me.

Saeedut Zafar Ali

Contents

Dedicated

to my supervisor and family members,

who offered me unconditional love and support.

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Getting an ideal teacher and kind supervisor, affectionate teachers, cooperative labmates, helping departmental members, loving and supportive family members are few of the countless blessings of Almighty ALLAH (SWT) has bestowed upon me. All praises for Him. Peace be upon His Prophet (S.A.W).

Abbreviations

1^{st} g	First generation of cephalosporins
2^{nd}g	Second generation of cephalosporins
3′CS	Three prime conserved sequence
3^{rd}g	Third generation of cephalosporins
4^{th}g	Fourth generation of cephalosporins
5′CS	Five prime conserved sequence
Ala	Alanine
Asp	Aspartic acid
Asn	Asparagine
Arg	Arginine
ATCC	American Type Culture Collection
bp	Base pair
CDC	Center for disease control and prevention
CI	Confidence interval
CLSI	Clinical and laboratory standards institute
CSF	Cerebrospinal fluid
CTX-M	Cefotaxime
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylene diamine tetra acetic acid
EMB	Eosine methylene blue
EPK	Extended spectrum β-lactamase producing *K. pneumoniae*
ERIC	*Enterobacterial repetitive intergenic consensus*
ESBL	Extended spectrum beta-lactamases
>	Greater than
≥	Greater than or equal to
Glu	Glutamic acid
Gln	Glutamine
Gly	Glycine
ICU	Intensive care unit
Ile	Isoleucine
IRT	Inhibitor resistant TEM
JNMC	Jawahar Lal Nehru Medical College
Kb	Kilo base pair
KPC	Klebsiella pneumonia carbapenemase
<	Less than
≤	Less than or equal to
LB	Luria-Bertani
Leu	Leucine
Lys	Lysine
µg	Microgram
µl	Micro litre

µg/ml	Microgram per milli litre
MDR	Multidrug resistance
MDRGNB	Multidrug resistant gram negative bacteria
MDROs	Multidrug resistant organisms
MIC	Minimum inhibitory concentration
Met	Methionine
mRNA	Messenger RNA
NDM-1	New Delhi metallo-β-lactamase
NICU	Neonatal intensive care unit
OMPs	Outer membrane proteins
OR	Odd ratio
ORFs	Open reading frames
OXA	Oxacillnase
PBPs	Penicillin binding proteins
%	Percent
PCR	Polymerase Chain Reaction
Pro	Proline
RNA	Ribonucleic acid
rpm	Revolution per minute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
Ser	Serine
Spp	Species
SHV	Sulfhydryl variable
TEM	Temoniera
Thr	Threonine
Tyr	Tyrosine
UTI	Urinary tract infection
Val	Valine
WHO	World health organization

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Abstract

Antibacterial resistance is a natural biological phenomenon of response of bacteria to the selective pressure of an antibiotic. The overuse and misuse of antibiotics in humans and in animal husbandry has been cited as a responsible factor in the development of drug resistance in all bacterial species. Emergence of multidrug resistance is more worrisome in entire medical fraternity. Multidrug-resistant strains possessing extended spectrum β-lactamases (ESBLs) has become an increasing problem worldwide. The advancing age, female gender, low socio economics status, illiteracy, hospital cross infection, the food chain trade and human migrations have contributed to increase the risk for community-acquired ESBL. CTX-M, SHV, TEM, and OXA enzymes have become the most prevalent ESBLs. The work presented in this thesis has explored the susceptibility and resistance pattern of clinical isolates of hospital setting, risk factors for acquisition of these organisms, mode of spread of resistance, prevalence of ESBL producers among bacterial isolates of clinical origin and their molecular mechanism of resistance.

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ampicillin and co-trimoxazole but sensitive to imipenem and meropenem. ESBL and MBL production was seen 7.3% and 12.2% of *E. coli* isolates, respectively. Three isolates were found to have $bla_{CTX\text{-}M\text{-}15}$ and two of them also showed $bla_{TEM\text{-}1}$ type enzymes. Whereas, none of them was observed to possess bla_{SHV}. Conjugation experiments using J-53 cells confirmed these resistant markers as plasmid mediated. The study highlights the molecular epidemiology of escalating antimicrobial resistance and likely switch over of $bla_{CTX\text{-}M\text{-}15}$ type extended spectrum β-lactamases by bla_{TEM} type ESBLs in India. Further, the antimicrobial resistance by horizontal gene transfer was predominant among *Enterobacteraceae* in the community setting.

Second chapter of this study was designed to (i) find out the susceptibility of antimicrobial agents, (ii) identify the various risk factors associated with ESBL producing *K. pneumoniae* clinical isolates, and (iii) understand the mode of mechanism among the *K. pneumoniae* isolates circulating in neonatal intensive care unit (NICU), Aligarh hospital of North India. Clinical isolates were characterized to detect *K. pneumoniae.* Susceptibility testing was done by standard disc diffusion method. MIC was determined using agar dilution methods. ERIC-PCR and resistant marker detection was performed by PCR amplification. Plasmid analysis and transfer experiments were carried out to further type the strains. Sequence analysis of resistant marker ($bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV}) was performed. Incompatibility and multilocus sequence typing were carried out. Among the clinical isolates, 103 (37.62%) strains were identified as extended spectrum β-lactamase producing *Klebsiella pneumoniae* (EPK) while remaining 262 (71.78%) strains were non-EPK. Furthermore, 103 EPK strains were characterized according to socioeconomic status, educational status, gestational age, onset sepsis, birth weight, maternal intrapartum

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The fourth chapter of this study aimed at (i) identifying the MBL producing bacterial isolates in infected diabetic foot ulcerated patient, (ii) determining and quantifying the resistance and susceptibility of those isolates, and (iii) characterizing the mode of transmission of CTX-M, TEM, SHV, NDM-1, VIM, OXA and Arm-A among the MBL producing clinical isolates. Twenty clinical isolates of infected diabetic foot patients from ICU and endocrinology ward of a teaching hospital of North India were screened for MBL producing pathogens. Antimicrobial susceptibility and MBL detection were performed and reconfirmed with HiComb MIC testing strips and disk diffusion method. MIC was determined using agar dilution methods. PCR amplification and sequence analysis of $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV}, $bla_{OXA\text{-}1}$, and Arm-A were carried out. To understand the mode of transfer of resistant marker, conjugational transfer method was used. Replicon typing and MLST were performed to type the strains. Insertion sequence was also identified. Out of 20 clinical isolates of diabetic foot ulcer, 14 strains were characterized as *K. pneumoniae*, 5 were *E. coli* and one was *E. cloacae*. One *K. pneumoniae* (Kp12) and one *E. cloacae* (Ec15) were also found to be carbapenem

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Chapter-1

Review of literature

1.1 Antibiotics:

1.1.1 Definition and characteristics of antibiotics

Antibiotics are antibacterial substance produced by various species of microorganism that suppress the growth of or kill other microorganism at very low concentration and have no or minimal effect on recipient. Common usage often extends the term antibiotics to include synthetic antimicrobial agents, such as sulfonamides and quinolones that also inhibit microorganism. Antibiotics differ markedly in physical, chemical, and pharmacological properties, in antimicrobial spectra, and in mechanism of action. Knowledge of molecular mechanism of bacterial replication has greatly facilitated rational development of compounds that can interfere with their replication.

1.1.2 Historical perspective

Antimicrobial drugs are greatest contribution of the 20th century to therapeutics. In earlier times, peoples were using indigenous substances in the treatment of infective disease, but both, physicians and patients, were unaware with the basis and mechanism of action of therapeutic agents. Ancient medicines have a long list of anti-protozoa drugs to cure the protozoan diseases and few for bacterial diseases like mercury and its compound for syphilis. Discoveries of microbes mediated pathology explored the pathway for its elimination in the terms of antimicrobial agents. In early 17th century, extract of cinchona bark (quinine) and ipecacuanha root (emetine) were identified and used as anti-malarial and anti-amoebic agents respectively [Garrod *et al.*, 1971; Greenwood *et al.*, 2000]. Paul Ehrlich, in early nineteenth century, has reported that dyes due to their differential affinities for various tissues may be used as antimicrobial agent. In 1904, the work of Ehrlich and Shiga has attracted the entire scientific world as they explored the usefulness and effectiveness of red dye called trypanrot against trypanosomes. [Mitsuhashi *et al.*, 1993]. In 1909, Ehrlich along with Sahachiro Hata observed that arsphenamine (named Salvarsan) is active against spirochetes and, therefore, very soon it become an effective anti syphilitic agent [Greenwood *et al.*, 2000].

Sulphonamides were the first antimicrobial drugs which were discovered by Gerhard Domagk [Domagk *et al.*, 1935]. Mietzsch and Klarer of Bayer Company synthesized Prontosil red, a red dye that binds to a sulfonamide group and later it was found by Domagk [Domagk *et al.*, 1935] to be an effective agent against hemolytic streptococci in mice. [Garrod *et al.*, 1971; Greenwood *et al.*, 2000]. Though, in vitro studies, prontosil red was shown to have no antibacterial activity. But later prontosil red was found to be an effective antimicrobial agent as it split into its component dye and an active antibacterial molecule, sulfanilamide that was already in the public domain [Trefouel *et al.*, 1935].

Idea of using the microbes as therapeutic agent was not new among the scientific world. Penicillin was the first to be discovered as natural antibiotics. An extract of *Pseudomonas aeruginosa* known as pyocyanase was in use for the treatment of wounds. [Garrod *et al.*, 1971]. Raper and Fennel introduced [Raper *et al.*, 1946] a strain of *Penicillium chrysogenum* producing higher yiled of penicillin [Demain *et al.*, 1999].

Discovery of penicillin became a mile stone for the discovery of many antimicrobial agents. Soil microorganisms were also taken by Selman Waksman (1940) for discovery of antibiotic molecule [Greenwood *et al.*, 2000]. And one of his students in 1943 got success to find out the new antimicrobial agent, streptomycin [Schatz *et al.*, 1944]. In same period, Rene Dubos [Hotchkiss *et al.*, 1941] identified a new molecule gramicidin, the first antibiotic active against gram-positive bacteria. And shortly thereafter, chlortetracycline, chloramphenicol, and some other molecules were discovered [Garrod *et al.*, 1971]. Among the identified and discovered molecules, many of them were found to be toxic but these discoveries lead to the development of more rational molecules and within only 10 years, drugs comprising the major classes of antibiotics were identified and developed [Greenwood *et al.*, 2000]. Majority of these molecules were identified and isolated from antibiotics producing microorganisms of interesting and unusual sources like wounds, sewage, a chicken's throat, and a wet patch of wall in Paris [Garrod *et al.*, 1971]. Nalidixic acid, a synthetic drug, was the first of the quinolones to be identified and though it was not therapeutically important but modification of nalidixic acid led to the production of the highly effective fluoro-quinolones. Members of this class, such as ciprofloxacin, norfloxacin, enorfloxacin, gatifloxacin and ofloxacin, have become very

important in the treatment of diseases in both humans and animals [Mitsuhashi *et al.*, 1993].

In sixth decade of twentieths century, after the discovery of few antibiotics, drugs development of antibiotics become a merely chemical modifications of existing drugs. These modifications are found to be quite useful and are capable to enhanced killing of pathogens and have a broad spectrum action. They are also attributed with lesser side effects [Lipsitch *et al.*, 2002]. Recently, combination of drugs for antibiotic therapy is introduced by clinician to overcome the drug resistance problem. The approach in combination drug therapy seems to be permissible as it has different mechanisms of action and enhanced effectiveness.

1.1.3 Susceptibility and resistance of antimicrobial agents

Successful antimicrobial therapy of an infection ultimately depends on the concentration of antibiotic at the site of infection. This concentration must be sufficient to inhibit growth of the offending micro-organisms. If host defenses are intact and active, a minimum inhibitory effect such as that provided by bacteriostatic agents may be sufficient. On the other hand, if host defenses are impaired, antibiotic mediated killing may be acquired to eradicate the infection. The concentration of drug at the site of infection not only inhibits the organism but also remain below the level that is toxic to human cell. If this can be achieved, the microorganism is considered susceptible to the antibiotics. The achievable serum concentration for an antibiotic, guides of break point for designating a microorganism as either susceptible or resistant by in vitro susceptibility testing. However, the concentration at the site of infection may be considerably lower than achievable serum concentration (e.g. various fluids of the eye or CSF). Local factors e.g. low pH, high concentration, and anaerobic conditions may also impair drug activity. Thus, the drug may be effective or ineffective in such cases even though standardized *in vitro* test would likely report the microorganism as sensitive. Conversely, concentration of drug in urine may be much higher than those in plasma. Microorganism that might otherwise be considered "resistant" may be eradicated when infection is limited to urinary tract.

1.1.4 Antibiotic failure: the leading therapeutic challenge

The recent emergence of antibiotic resistance in bacterial pathogens, both nosocomially and in community acquired is very serious development that threatens the end of the antibiotic era. Today, more than 70% of the bacteria associated with hospital acquired infections in United States are resistant to one or more of the drugs previously used to treat them. Bacterial resistance renders therapy more costly and sometimes patients may succumb to resistant organism due to failure of all available antibiotics [Levy & Marshall., 2004]. Enteric disease agents such as *Salmonella enteritidis*, *Shigella flexneri* and *Vibrio* spp, especially, in developing countries, found to be multi drug resistant and become a challenge for public health. Penicillin-resistant strains of pneumococci account for 50% or more of isolates in some European countries, and the proportion of such strain is rising in the United States. Increasing pneumococci resistance led a worldwide threat in treatment of pneumonia and ear infections. Recent reports of microbial resistance to macrolides and tetracyclines exposed the susceptibility of these antimicrobial agents [Schrag *et al.* 2004]. The world wide emergence of *Haemophilus*, and *gonococci* that produce β-lactamase is major therapeutic problem. Methicillin-resistant strains of *staphylococcus aureus* are endemic in hospital and are isolated increasingly from community-acquired infections [Naimi *et al.*, 2003; Vandenesh *et al.*, 2003]. In US and UK, 40–60% of nosocomial *S. aureus* strains are found to be methicillin-resistant (MRSA), and usually MDR [Weinstein *et al.*, 2001; Anonymous *et al.*, 2002]. Higher mortality rate are usually associated with MRSA as compared to methicillin-sensitive strains [Cosgrove *et al.*, 2003]. Some MRSA also showed low level resistance to drug of choice vancomycin [Hiramatsu *et al.*, 1998, Fridkin *et al.*, 2001]. Acquired vancomycine resistant *S.aureus* strains from vancomycin resistant enterococci have been observed in US [Weigel *et al.*, 2003; Tenover *et al.*, 2004]. Some strains of methicilin and vancomycin-resistant *S. aureus* have also emerged as resistant to linezolid and the streptogramin combination, dalfopristin/quinopristin [Jones *et al.*, 1998; Meka & Gold *et al.*, 2004]. In developed countries, MRSA strains possessing a new virulence toxin (Panton-Valentine leukocidin) have been emerged as resistant to β-lactam antibiotics [Vandenesch *et al.*, 2003; Herold *et al.*, 1998]. The failures of antibiotics therapy in

MRSA infection render to bring an alternative therapy when MRSA is suspected as disease had become too far advanced [Anonymous *et al.*, 1999]. Multi-drug resistance strains of *S.aureus* with intermediate susceptibility to antibiotics and high level resistance to vancomycin have been reported [Hiramatsu *et.al.*, 1997; smit *et.al.*, 1999, weigel *et. al.*, 2003]. The emergence of multidrug resistant strains of Gram-negative bacteria (*Pseudomonas, Klebsiella, Enterobacter, Acinetobacter, Salmonella species,*) and Gram-positve organisms (*Staphylococcus, Enterococcus, Streptococcus species*) is more worrisome in the present therapeutic scenario. Among the *Enterobacteriaceae*, β-lactamases producers, *Enterobacter* and *Klebsiella* are found to inactivate the latest generation of penicillins and cephalosporins [Bush *et al.*, 2001; Bradford *et al.*, 2001]. Some strains of enterobacteriaceae bearing metallo-β-lactamases are also observed to be resistant to carbapenems [Nordmann & Poirel., 2002; Livermore & Woodford., 2000]. *E.coli* strains, a common cause of urinary tract infection, have been found to be 60-70% resistant to the majority of drug families including fluoroquinolones especially in Southeast Asia and China, and also in US and UK [Jones, R.N. *et al.* 1998; Meka, V.G. & Gold, H.S. 2004]. This trend of resistance jeopardizes the worth of this drug [Zervos *et al.*, 2003; Karlowsky *et al.*, 2002]. Many patients of tuberculosis are remained untreated due to resistant *M. tuberculosis* to all anti-tubercular drugs [Bloom & Murray., 1992]. Inadequate treatment of tuberculosis increases risk of bacterial resistance, and more than 50% of such individuals have MDR tuberculosis. Epidemics of multi drug resistance strains of *Mycobacterium tuberculosis* have been reported in the United States. Worldwide physicians are facing the problem of penicillins, tetracyclines and fluoroquinolones resistance in *N. gonorrhoeae* [Tanaka *et al.*, 2000; Anonymous *et al.*, 2002]. Parenteral cephalosporin is only treatment as a single dose therapy to infected individuals with this pathogen, but, a recent report showing the decreased susceptibility of cefixime to *N. gonorrhoeae* warns the future of this group of drugs [Wang *et al.*, 2003]. The study predicted about the encumbered community with MDR organism that, multidrug resistance will override single-drug resistance in the present decade [McCormick *et al.*, 2003]. Resistance problem in community indicates that resistant strains can be traced from the community to the hospital and vice versa, indicating that

drug resistance is no longer localized as resistance in the community and has extended the resistance beyond the hospital premises.

1.1.5 Classification and mechanism of action of antimicrobial agents

Antimicrobial agents are calssified based on chemical structure and proposed mechanism of action as follows: (1) agents that inhibit synthesis of bacterial cell walls, including the β-lactam class (e.g. penicillin, cephalosporins, and carbapenems) and dissimilar agents such as cycloserine, vancomycin, and bacitracin; (2) agents that act directly on the cell membrane of microorganism, increasing permeability and leading to leakage of intracellular compounds, including detergents such as polymyxin; polyene antifungal agents (e.g.amphotericin B) which binds to cell wall sterols; and the lipopeptide daptomycin [Carpenter and Chambers., 2004];

Table 1.1 Major classes of antibiotics

Protein synthesis	**Aminoglycosides**	**Chloramphenicol**	**Tetracyclines**	**Macrolides**	**Streogramins**
	Streptomycin			Erythromycin	Virginiamycin
	Neomycin			Azythromycin	Quinopristin
	Kanamycin			Clarithromycin	Dalfopristin
	Gentamicin				Pristinamycin
Nucleic acid synthesis	**Sulphonamides (Diaminopyrimidines)**			**Quinolones**	
	Sulfamethoxazole trimethoprim			Ciprofloxacin	
				Norfloxacin	
Cell wall synthesis	**β-lactams**			**Glycopeptides**	
	Penicillins			Vancomycin	
	Cephalosporins			Avopuricin	
	Carbapenems				

(3) agents that disrupt function of 30S or 50 S ribosomal subunit to reversibly inhibit protein systhesis, which generally are bacteriostatic (e.g. choloamphenicol, the tetracyclines, erythromycin, streptogramins, clindamycin and linezolids);(4) agents that binds to 30S ribosomal subunit and later protein systhesis, which genrally are bactericidal (e.g. aminoglycosid); (5) agents that affect the bacterial nucleic acid metabolism, such as the rifamycin (e.g. rifampin and rifabutin), which inhibit RNA polymerase, and the quinolones, which inhibit topoisomerases and (6) the antimetabolites, including trimethoprim and sulfonamides, which block essential enzymes of folate metabolism. But broadly it may be classified and understood under following three major groups (Table-1.1).

a. Inhibition of cell wall synthesis

β-lactams and glycopeptides are most important bacterial cell wall synthesis inhibitors. They are frequently prescribed antimicrobial agents sharing a common structure and mechanism of action i.e. inhibition of bacterial peptidoglycan cell wall synthesis. Since, bacterial cell walls are wholly unlike the membranes of eukaryotes, therefore, they are an obvious target for selectively toxic antibiotics. β-lactams includes penicillins, cephalosporins, and the carbapenems. These agents bind to the penicillin binding proteins (PBP's) that cross-link strands of peptidoglycan in the cell wall. In gram negative cells, this leads to the formation of fragile spheroplasts that are easily ruptured. In gram positive cells, autolysis is triggered by the release of lipoteichoic acid.

Chromosomally encoded β-lactamases enzymes are found in many bacteria that are necessary for cell wall production. But, when production of these enzymes increases, it inactivates β-lactam antibiotics by hydrolyzing of β-lactam ring and thus producing the resistance [Greenwood *et al.,* 2000]. β-lactamases encoded by plasmids or other transmissible elements can lead to such overproduction and, therefore, to resistance [Normark & Normark, 2002]. Alteration in PBPs may also results in reduced penicillin binding and, hence, resistant [Greenwood *et al.,* 2000]. After the discovery of penicillin and bacterial resistance, many new molecules of the β-lactams have been developed and clinically used. These new molecules having different spectrums of activities have

different susceptibility to β-lactamases. Clavulanic acid has the ability to bind irreversibly to β-lactamases and, thereby, inhibit their action. Drug combinations of this type of molecule with β-lactam have been proved useful in treatment of several pathological conditions [Bryan *et al.*, 1984].

Glycopeptides are a group of antibiotics that include vancomycin, avoparcin etc, which bind to acyl-D-alanyl-D-alanine. Binding of this compound prevents the addition of new subunits to the growing peptidoglycan cell wall, thus limiting their action to gram positive organisms. Earlier, resistance to glycopeptide was thought to be rare, but has recently been shown to be quite common [Bryan *et al.*, 1984]. Resistance in *Enterococci* has developed through newly discovered enzymes that use D-alanyl-D-lactate in place of acyl-D-alanyl-D-alanine, allowing cell wall synthesis to continue. Other mechanisms of resistance involve the over-production of peptidoglycan precursors which overwhelm the drug [Greenwood *et al.*, 2000].

b. Inhibitors of protein synthesis

Various groups of antibiotics are responsible for inhibition of bacterial protein synthesis. The aminoglycoside groups is broadly divided into three major groups; streptomycins, neomycin, and kanamycin. Through active transport system in bacteria, antibiotics enter in bacterial cells with the help of quinolones that are absent in anaerobes and streptococci, making them unavailable for pharmacological action of these agents. Aminoglycosides irreversibly binds to 16S rRNA and block the initiation complex. By binding to 16S rRNA the aminoglycosides increase the affinity of the A site for tRNA regardless of the anticodon specificity. Streptomycins have the affinity to bind with the 30S ribosomal subunit while kanamycin and neomycin with 50S subunit as well as with sites on 30S subunit in unique manner that is different from streptomycin and thus produce their effect [Greenwood *et al.*, 2000]. Drugs after binding with their sites, start their activity by involving initiation complexes and cell membrane proteins that contribute to cell death and plays a role in the action of these antibiotics, but this is poorly understood [Bryan *et al.*, 1984; Greenwood *et al.*, 2000].

Aminoglycoside resistance mechanism include: (a) the deactivation by N-acetylation, adenylation or O-phosphorylation (b) the reduction of the intracellualar concentration of aminoglycosides by changes in outer membrane permeability, decreased inner membrane transport, active efflux, and drug trapping, (c) the alteration of 30S ribosomal subunit target by mutation, and (d) methylation of the aminoglycoside binding site [Shakil *et al.*, 2008; Bryan *et al.*, 1984].

Chloramphenicol, naturally occurring from *Streptomyces venezuelae*, is a broad-spectrum antibiotic and produced by chemical synthesis. Chloramphenicols inhibit protein synthesis in bacteria and to lesser extent in eukaryotic cell. It also inhibits peptide bond formation on 70S ribosomes [Bryan *et al.*, 1984]. This drug is especially useful as it can penetrate bacterial cell, eukaryotic cells and cerebrospinal fluid probably by passive diffusion, making it a drug of choice for treatment of meningitis and intracellular bacterial infections. But its use is very limited due to fatal side-effect i.e aplastic anemia [Greenwood *et al.*, 2000].

Resistance to chloramphenicol usually is caused by a plasmid encoded chloramphenicol acetyltransferase enzyme that inactivates the drug. A number of these enzymes have been discovered, each altering the chloramphenicol molecule to prevent binding to the bacterial ribosome. Resistance to cholramphenicol in gram negative cell can also results from decreased permeability due to alteration in outer membrane that prevent the drug from entering the cell and also from ribosomal mutation [Bryan *et al.*, 1984].

The tetracyclines are another group of broad-spectrum antibiotics that inhibit bacterial protein synthesis by binding to the 30S bacterial ribosome and preventing access of aminoacyl tRNA [Roberts *et al.*, 1996]. They are brought into the cell of gram-negative bacteria by passive diffusion through hydrophilic channels formed by the porin proteins of the outer cell membrane and by active transport that pumps all tetracyclines across the cytoplasmic membrane. Entry of these drugs in to gram-positive bacteria requires metabolic energy but it is not well understood.

Resistance to tetracycline is often plasmid mediated and often is inducible. The three main mechanisms are: (1) production of a membrane efflux pump that removes the drug

as rapidly as it enters and there are several genes encoding these pumps; (2) production of several ribosomal protection proteins that displace the tetracycline from binding to the ribosome, (3) a protein found only in Bacteroides spp., enzymatically inactivates tetracycline [Roberts *et al.*, 1996]. Interestingly, efflux pump inhibitors have recently been discovered that may allow combinations of these inhibitors and tetracyclines to be used against previously resistant strains [Chopra *et al.*, 2002].

Macrolides antibiotics contain a many-membered lactone ring to which are attached one or more deoxy sugar. They are commonly used to treat gram positive and intracellular bacterial pathogens. Erythromycin was the first of these, and several other important macrolides have been discovered since, including clarithromycin and azithromycin. It was originally believed that erythromycin inhibited protein synthesis by competing with amino acids for ribosomal binding sites, but newer research shows that several mechanisms are involved [Garrod *et al.*, 1971]. The macrolides are now believed to promote dissociation of tRNA from the ribosome, inhibit peptide bond formation, inhibit ribosome assembly, and prevent amino acid chain elongation [Gaynor *et al.*, 2003].

Resistance to macrolides usually results from one of four mechanisms: (1) drugs efflux by an active pump mechanism that removes the drug from the cell; (2) modification of the ribosome by inducible or constitutive production of methylase enzymes that decrease the drug binding; (3) macrolide hydrolysis by easterases produced by *Enterobacteriaceae*; and (4) chromosomal mutation that alter 50S ribosomal protein and allosterically prevent macrolide binding. A common alteration is dimethylation of one nucleotide on the 23S rRNA that does not only prevents macrolide binding, but also confers resistance to lincosamide and streptogramin antibiotics [Gaynor *et al.*, 2003].

The **streptogramins** are another class of protein synthesis inhibitors that inhibits protein synthesis mostly in gram positive organisms probably due to decreased permeability of their outer membrane. These antibiotics are actually combinations of structurally different drugs, types A and B that act synergistically. These compounds bind to separate sites on the 50S subunit. Type A drugs block attachment of substrates at two sites on the 50S subunit, whereas type B drugs cause release of incomplete protein chains. The synergistic effect arises from a conformational change induced by the binding of a type A

drug which significantly increases affinity of type B drugs [Johnston *et al.*, 2002]. Streptogramins which are currently in use include virginiamycin, pristinamycin, and quinupristin/dalfopristin.

Resistance to streptogramin antibiotics can be found in several forms. Efflux pumps for both type A and B streptogramins have been identified. Type A streptogramins can be inactivated by one of the virginiamycin acetyl-transferases, and several enzymes have been identified that can inactivate type B streptogramins. Alteration of bacterial ribosomal proteins or RNA can also confer resistance. A common mutation is the dimethylation of one nucleotide on the 23S rRNA, mentioned previously, that gives rise to resistance to type B drugs, as well as macrolides and lincosamides [Johnston *et al.*, 2002].

c. Inhibitors of nucleic acid synthesis

The introduction of diaminopyrimidines in combination with sulfonamide constitute an important advance in the development of clinically effective antimicrobial agents and represents the practical application of a theoretical consideration; i.e if two drugs act on sequential steps in the pathway of an obligate enzymatic reaction in bacteria, the result of their combination will be synergistic. Although both types of drugs are useful on their own, they exhibit a synergistic effect when combined. Sulfonamides are currently not used commonly in medicine, but the combination drug trimethoprim-sulfamethoxazole is sometimes used in the treatment of urinary tract infections. The sulfonamides and the diaminopyrimidines indirectly inhibit the nucleic acid synthesis by inhibiting folate systhesis. Folate is a coenzyme required in synthesis of purines and pyrimidines. Sulfonamides serve as an analog of p-aminobenzoic acid. Therefore, they competitively inhibit an early step in folate synthesis. Diaminopyrimidines, of which trimethoprim is the most common, inhibit dihydrofolate reductase, the enzyme that catalyzes the final step in folate synthesis [Greenwood *et al.*, 2000].

Microorganisms employ several resistance mechanisms against each of the anti-folate drugs. For example, sulfonamides are rendered ineffective by over-production of p-aminobenzoic acid or production of an altered dihydropteroate synthetase. The substrate

for dihydropteroate synthetase is p-aminobenzoic acid, and the altered form has a much lower affinity for sulfonamides than for p-aminobenzoic acid [Then *et al.*, 1982]. Trimethoprim resistance can also result from several mechanisms, e.g., over-production of dihydrofolate reductase or production of an altered, drug-resistant form can lead to resistance [Bryan *et al.*, 1984]. In addition, both drugs can be enzymatically inactivated, resulting in resistance [Then *et al.*, 1982].

The quinolones; ciprofloxacin, norfloxacin, moxifloxacin, gatifloxacin and nalidixic acid, are a chemically varied class of broad-spectrum antibiotics widely used to treat many diseases, including gonorrhea and anthrax. Among the quinolones, ciprofloxacin is more used in clinical practices than any other antibacterial agent [Acar *et al.*, 1997].

Quinolones antibiotics target bacterial DNA gyrase and topoisomerase IV, which are necessary for correct functioning of supercoiled DNA and thus inhibit their bacterial growth [Greenwood *et al.*, 2000]. In many gram positive organisms, topoisomerase IV is inhibited by the quinolones. In contrast, for many gram negative bacteria, DNA gyrase is the primary quinolone target [Ruiz *et al.*, 2003].

Several mechanisms of resistance to quinolones have been described. However, three most important mechanisms of resistance to quinolones have been established. Decreased expression of membrane porins leads to resistance in some quinolones. Cross-resistance to other drugs requiring these porins for activity also results from these changes. A second mechanism of resistance to quinolones is expression of efflux pumps in both gram negative and gram positive organisms [Novak *et al.*, 1999]. And the third is the alteration of the target enzymes. Several mutations in bacterial chromosomal genes encoding DNA gyrase or topoisomerases IV has also been reported that may result in reduced binding affinities [Ruiz *et al.*, 2003]. It is believed that series of successive mutation in the target genes, rather than single mutation, is responsible for high-level quinolone resistance [Novak *et al.*, 1999].

1.2 Drug resistance

1.2.1 Mechanisms of antibiotics resistance

Structural difference of eukaryotic and prokaryotic cells at cellular level is the key factor for differential selective toxicity of antibiotics and this structural difference among bacterial species can lead to resistance to certain antibiotics. The differences between intrinsic and acquired resistance to an antibiotic are also important for research as well therapeutic purposes. Intrinsic resistance that indicates the resistance of entire species to antibiotics is only because of inherent characteristics requiring without any genetic alteration. Many organisms are intrinsically resistant to a particular drug or molecule because they lack the target site of that durg or molecule. For example, Mycoplasma are always resistant to β-lactam antibiotics as they are lacking of peptidoglycan (which the β-lactams act upon). Many drug molecules, in case of gram negative organism, are unable to reach their target site. *Pseudomonas aeruginosa* has drug efflux pump and thus restrict the outer membrane permeability and thereby exhibiting high intrinsic resistance to many antibiotics. Acquired Resistance that arises through mutation or horizontal gene transfer is well established phenomenon in drug resistance. Generally, bacterial resistance to antimicrobial agent is attributed to three mechanisms; (1) the drug does not reach its target, (2) the drugs are not active, or (3) the target is altered [Davies *et al.*, 1994; Spratt *et al.*, 1994; Li & Nikaido, 2004]. The outer membrane of gram negative bacteria is a permeable barrier that excludes large molecules from entering the cell. Small polar molecules, including many antibiotics, enter the cell through protein channel called porins. Absence of mutation, or loss of favored protein channel can slow the rate of drug entry into cell or prevent entry altogether, effectively reducing drug concentration at target site. If the target is intracellular and the drug requires active transport across the cell membrane, a mutation or phenotypic change that shut down this transport mechanism can confer resistance as observed in the case of gentamicin resistance. Bacteria also have efflux pumps that can transport drugs out of the cell. Resistance to numerous drugs, including tetracycline, chloramphenicol, fluoroquinolone, macronilides, and β-lactam antibiotics, is mediated by an efflux pump mechanism [Li & Nikaido, 2004]. Drug

inactivation is the second general mechanism of drug resistance. Bacterial resistance to aminoglycosides and to β-lactam antibiotics usually is due to production of an aminoglycoside-modifying enzyme or β-lactamase, respectively. A variation of this mechanism is failure of the bacterial cell to activate a prodrug. This is the basis of the most common type of resistance to isoniazide in *M. Tuberculosis* [Bertrand *et al.*, 2004]. The third general mechanism of drug resistance is target alteration. This may be due to mutation of natural target (e.g. Fluoroquinolne resistance), target modification (e.g. ribosomal protection type of resistance to macronilides and tetracyclines), or acquisition of resistant from the native, susceptive target (e.g. staphylococcal methicillin resistant caused by production of low-affinity penicillin-binding protein) [Nakajima *et al.*, 1999; Hooper *et al.*, 2002; Lim and Strynadka, 2002]. Drug resistance may be acquired by mutation and selection, with passage of trait vertically to daughter cell. For mutation and selection to be successful in generating resistance, the mutation cannot be lethal and should not appreciably alter virulence. For the trail to be passed on the original mutant or its progeny also must disseminate and replicate; otherwise, the mutation will be lost until it is rediscovered by some other mutant arising from within a wild type population. Drug resistance more commonly is acquired by horizontal transfer of resistance determinants from donor cell, often to another bacterial species, by transduction, transformation, or conjugation. Resistance acquired by horizontal transfer can disseminate rapidly and widely either by colonal spread of the resistant strain or by subsequent transfer to other susceptible recipient strains. Horizontal transfer of resistance offers several advantages over mutation selection. Firstly, the lethal mutation of an essential gene is avoided and the level of resistance in horizontal gene transfer is higher than that produced by mutation. Secondly, the changes acquired in the gene can still be transmitted vertically and can be mobilized and rapidly amplified within population by transfer to susceptible bacteria. Thirdly, the resistance gene can be eliminated when it no longer offers selective advantage to the organism [Murray *et al.*, 1992].

1.2.2 Mutation selection

Mutation is the molecular basis for resistance to streptomycin (ribosomal mutation), quinolone (gyrase or totpisomerase IV gene mutation) rifampin (RNA polymerase gene mutation), and linezolid (ribosomal RNA mutation). This mechanism underlies all drug resistance in *M. Tuberculosis* [Riska *et al.*, 2004]. Mutation may occur in the gene encoding (1) the target protein, altering its structure so that it no longer binds the drug; (2) a protein involved in drug transport; (3) a protein important for drug activation or inactivation, in the case of extended spectrum β- lactamase [Bush *et al.*, 2001]; or (4) in a regulatory gene or promoter affecting expression of the target, a transport protein, or an inactivating enzyme. Mutations are not caused by drug exposure *per se*. Mutations are random events that confer a survival advantage to the organism when drug is present. However, certain drugs that induce the bacterial SOS system of DNA repair protein that accommodate potentially lethal stress (*e.g.* fluoroquinolone) may facilitate resistance gene transfer or increase the mutation frequency by induction of error-prone polymerases [Goodman *et al.*, 2002; Chopra *et al.*, 2003; Beaber *et al.*, 2004]. Any large population of antibiotic-susceptible bacteria is likely to contain rare mutants that are less susceptible than the parent. Through, sequential acquisition of more mutants, clinically siginificant resistance may emerge. High level resistance of *E.coli* to fluoroquinolone is due to accumulation of multiple additive mutions. In some instance, a single-step mutation results in a high degree of resistance. For example, a point mutation within the drug binding domain in the B subunit of bacterial RNA polymerase confers high level resistance to rifampin.

1.2.3 Horizontal gene transfer

Horizontal transfer of resistance genes is greatly facilitated by and is largely depend on the mobile genetic elements. The role of plasmid and transducing phages as carriers of resistance gene and transfer elements is discussed in more details below. Other mobile elements, transposable elements, integrons and genetic cassettes also participate in the process. Transposable elements are of three general types: insertion sequence,

transposons, and transposable phages; two of these, insertion sequence and transposon are important for resistance. Insertion sequence [Mahillon & Chandler.,1998] are short segment of DNA encoding enzymatic function (e.g. transposases and resolvases) for site specific recombination with inverted repeat sequence at either end. They can copy themselves as insert themselves into the chromosomes or a plasmid. Insertion sequence does not encode resistance, but they function as sites for integration of other resistance-encoding elements, e.g. plasmid or transposons.

(a) Trasnsposons are basically insertion sequence that also code for other functions, one of which can be drug resistance. Since transposons move between chromosome and plasmid, the resistance gene can hitchhike its way onto a transferable element out of the host and into recipient. Transposons are mobile element that excise and integrate in bacterial genomic or plasmid DNA (i.e. from plasmid to plasmid, plasmid to chromosome, or from chromosome to plasmid).

(b) Integrons are not formally mobile and do not copy themselves, but they encode an integrase and provide a specific site into which mobile gene cassettes can be integrated. Gene cassettes encode resistance determinants, usually lacking a promoter, with downstream repeat sequence. The integrase recognizes this repeat sequence and direct insertion of cassettes into position behind a strong promoter that is present on integron. Integrons may be located within transposons or in plasmids, and therefore may be mobilized, or located on the chromosome [Fluit & Schmitz., 2004]. Another type of gene cassettes, SCCmec (Staphylococcal Chromosomal Cassettes), has been described in methicilline-resistant strains of staphylococci [Katayama *et al.*, 2000]. The methicllin resistance gene mecA is located within this cassette along with recombinase gene. The recombinases both excise and integrate the cassette element, which exist as a circular intermediate that is not self replicating, into a very specific site in the staphylococcal chromosome. How this element is transferred and the role of exicision-mobilisation in this process is not known.

(c)Transduction is process of acquisition of bacterial DNA from a phage (a virus that propagates in bacteria) that has incorporated DNA from a previous host bacterium within its outer protein coat. If the DNA includes a gene for drug resistance, the newly infected

bacterial cell may acquire resistance. Transduction is particularly important in the transfer of antibiotics resistance among strains of *S. aureus.*

(d) Transformation is defined as uptake of DNA into the host genome by homologous recombination of free DNA released into the environment by other bacterial cells. Transformation is the molecular basis of penicillin resistance in *Neisseria* [Spratt *et al.*, 1994]. Penicillin-resistance penumococci produce altered penicillin binding proteins (PBSs) that have low affinity binding of penicillin. Nucleotide sequence analysis of the genes encoding these altered PBSs indicates that they are mosaics in which blocks of foreign DNA from a closely related species of streptococcus have been imported and incorporated into the resident PBP gene.

(e) Conjugation is phenomenon of direct gene transfer through cell to cell contact by sex pilus or bridge. This complex and fascinating mechanism for spread of antibiotics resistance is extremely important because multiple resistance genes can be transferred in a single event. The transferable genetic material consists of two different sets of plasmid encoded genes that may be on the same or different plasmids. One set encodes the actual resistance; the second encodes genes necessary for the bacterial conjugation process.

Conjugative plasmids tend to be rather large (≥50 Kb). The combine element of plasmid DNA rolling-circle replication (only a single strand is transferred, and it replicates in the host) with a type of IV bacterial secretion system. Plasmid transfer requires an origin of transfer demarcating the site within the plasmid where transfer will occur, DNA replicating enzymes, and coupling proteins that direct the DNA across two cell membranes on its way from the host into the recipient. Genes encoding the resistance determinants may be located transposons.

Conjugation with genetic exchange between nonpathogenic and pathogenic microorganisms probably occurs in the gastro intestinal (GI) tract of human beings and animals. The efficiency of transfer is low; however, antibiotics can exert a powerful selective pressure to allow emergence of restraint strain. Genetic transfer by conjugation is common among gram negative bacilli, and resistance is conferred on susceptible cell as a single event. *Enterococci* also contain a broad range of conjugative plasmid that are

involved in the transfer and spread of resistance genes among gram positive organism. Vacomycin resistance in *Enterococci* is mediated by a conjugative plasmid [Arthur and Courvalin, 1993; Murray *et al.*, 20000]. Vancomycin resistance in *S. aureus* is due to conjugative transfer of vanA-type vancomycin resistance genes encodes on a transposon from *Enterococcus fecalis* donor into methicillini-resistant strain of *S. aureus* with subsequent integration of the transposon into a resident staphylococcal conjugative plasmid.

(f) Plasmids, a double stranded circular or linear DNA molecule capable of autonomous replication, are usually found in prokaryotes, although they exist in some groups of primitive eukaryotes as yeast, fungi and cellular slime moulds [Rush *et al.*, 1985]. They are not organisms as they thought to be extra-chromosomal, accessory DNA elements [Reanney *et al.*, 1976, Campbell *et al.*, 1981]. Normally, Plasmids do not possess such genes that are essential for growth of their host in normal condition. Natural plasmids are able for their autonomous replication, controlling the copy-number and ensuring stable inheritance during cell division and many of them, through the conjugation process, can promote their horizontal transfer among bacteria of different genera and kingdoms. Extensive researches have been done to find out the role of plasmid in bacterial genome [Thomas *et al.*, 2005]. Plasmids carry genes that confer a selectable phenotypic character under specific niche conditions. Antimicrobial resistance is best example of phenotypic advantage of plasmid [Lenski *et al.*, 1998]. Resistance genes on plasmids give immediate offer to their host under antimicrobial therapy. Though, the real advantage of the plasmid location of resistance genes is not well established: transfer of plasmid-located resistance genes to the bacterial chromosome may disfavor the plasmid maintenance. Advantage of plasmid location for selectable genes may be hypothesized in the relative higher copy number of plasmids by increasing the gene dosage. But there are numerous other factors influencing the chromosome/plasmid harmony and evolution. Plasmids must be thought a selfish molecule that is autonomous in nature and encode extra genetic information, establish the complex relationships with the recipient host and play multiple roles in the bacterial population. Antimicrobial resistance arises from a complex multi-factorial process supported by panoply of mobile genetic elements that contain and transfer resistance determinants. Resistance genes located on plasmids move from one bacterium

to another, conferring phenotypic characteristics. Several resistance plasmids have been identified and described to carry virulence factors, such as bacteriocins, siderophores, cytotoxins, or adhesion factors [Martinez *et al.*, 2002] and virulence plasmids have been described to carry resistance genes [Guerra *et al.*, 2002, Villa *et al.*, 2005, Herrero *et al.*, 2006]. For plasmid carrying virulence and resistance linked determinants, an infective population will be selected for antimicrobial resistance, and antimicrobial resistance pressure will select the virulence traits. However, once those determinants have been selected in the bacterial host, they can evolve further and eventually be transferred to other bacterial population. The acquisition of antimicrobial resistance genes on virulence plasmids could represent a novel tool in bacterial evolution, implementing adaptive strategies to explore and colonize novel hosts and environments [Martinez *et al.*, 2002]. Plasmid can code for more than one β-lactamase in addition to their expression of chromosomal enzyme. Due to carriage of plasmids and promiscuous exchange of such material between bacteria, these resistance genes have spread widely and are also subject to mutations [Lee, Yuen & Kumana., 2001]. Plasmid mediated β-lactamases were first recognized in Gram-negative bacteria in the early 1960s, shortly after the introduction of ampicillin [Livermore *et al.*, 1993]. Plasmid-mediated resistance to β-lactam antibiotics in *E. coli* is of major concern in hospitals. A widespread resistance of *E. coli* to ampicillin and co-trimoxazole has been reported in South Africa [Klugman *et al.*, 1993]. It has been reported that mortality and morbidity in prolonged hospitalization are twice as high in patients infected with antibiotic-resistant strains than with susceptible strains [Pitout *et al.*, 1997; Silva *et al.*, 1999; Lautenbach *et al.*, 2001].

1.2.4 Multidrug resistance (MDR) in bacteria

Multi-drug resistant organisms may be defined as bacteria with simultaneous resistance to three or more different classes of antibiotics [Guyot *et al.*, 1999]. The rise in multi drug resistance organism attracted the world wide biomedical researchers. In a recent study in North America, it was found that more than 55% *E.coli* of fluoroquinolone resistant uropathogens were additionally resistant to ampicillin as well as trimethoprim-sulfamethoxazole (TMP/SMX) [Zhanel, Unpublished data]. And it was also observed that

drug resistance cases were more common in those patients who had history of prolonged usage of antibiotic. Multi drugs resistant organism causes a higher degree of treatment failure and morbidity associated with infection [Gupta & Stamm., 2002; Sobel & Kaye., 2004].

1.3 β-Lactamases

1.3.1 β-Lactamase: Definition, characteristics and classification

β-lactamases are categorized on the basis of similarity in amino acid sequence (Ambler classes A through D) or on substrate and inhibitor profile (Bush-Jacoby-Medeiros Groups 1 through 4) (Table 1.2) [Ambler *et al.*, 1980; Bush *et al.*, 1995; Huovinen *et al.*, 1991; Jaurin *et al.*, 1981; Ouellette *et al.*, 1987]. They are globular proteins that characteristically have α-helices, β-pleated sheets, and share similar structural features. Bush Group 2 enzymes (Ambler Class A) known as penicillinases are susceptible to β-lactamases inhibitors. The TEM-1 and SHV-1 β-lactamases, Group 2b, are the β-lactamases usually found in *K. pneumoniae* and *E. coli* that confer resistance to penicillins (ampicillin and piperacillin) [Bush *et al.*, 1995]. Bush Group 3 enzymes (Ambler Class B) are MBLs that use one of two zinc (Zn2+) atoms for inactivating penicillins and cephalosporins. In bacteria, MBLs are among the most resistant phenotypes encountered by clinicians and confer resistance to carbapenems, cephalosporins and penicillins. MBLs are inhibited by chelating agents (EDTA), but not by clavulanic acid or sulfones. The bla genes encoding MBLs are found on a variety of genetic elements (chromosome, plasmid, integrons, etc.) [Walsh *et al.*, 2005]. IMP-type and VIM-type of *P. aeruginosa* possessing MBLs are most widespread and clinically important and around 20% of imipenem-resistant *P. aeruginosa* strains have been reported possessing a MBL. Bush Group 1 enzymes (Ambler class C) include the chromosomally encoded AmpC type β-lactamses nad has been observed in *Citrobacter freundii*, *Enterobacter aerogenes* and *Enterobacter cloacae*, *Morganella morganii*, *P. aeruginosa* and *Serratia marcescens*.

Table 1.2 β-lactamase classification by Bush and Jacoby

Ambler class	Bush-Jacoby group	Distinctive substrates	Inhibited by		Representative enzymes
			CA / TZB	EDTA	
C	1	Cephalosporins	-	-	AmpC, P99, ACT-1,CMY-2, FOX-1,MIR-1
C	1e	Cephalosporins	-	-	GC-1, CMY-37
A	2a	Pencillins	+	-	PC1
A	2b	Pencillins, early cephalosporins	+	-	TEM-1, TEM-2, SHV-1
A	2be	Extended-spectrum cephalosporins, monobactams	+	-	TEM-3, SHV-2, CTXMs,PER, VEB
A	2br	Penicillins	-	-	TEM-30, SHV-10
A	2ber	Extended-spectrum cephalosporins, monobactams	-	-	TEM-50
A	2c	Carbencillin	+	-	PSE-1, CARB-3
A	2ce	Carbencillin, cefepime	+	-	RTG-4
D	2d	Cloxacillin	V	-	OXA-1, OXA-10
D	2de	Extended-spectrum cephalosporins	V	-	OXA-11, OXA-15
D	2df	Carbapenems	V	-	OXA-23, 0XA-48
A	2e	Extended-spectrum cephalosporins	+	-	CEPA
A	2f	Carbapenems	V	-	KPC-2, IMI-1, SME-1
B	3a (B1)	Carbapenems	-	+	IMP-1, VIM-1, IND-1, CcrA
	(B2)				L1, CAU-1, GOB-1,FEZ-1
B	3b (B3)	Carbapenems	-	+	CphA, Sfh-1
Unkown	4	-			Mostly no yet sequenced

(V), Variable; (+), Yes; (-), No; CA, Clavulanic acid; TZB, Tazobactam

Penicillins, β-lactamase inhibitors, cefoxitin, cefotetan, ceftazidime, ceftriaxone, and cefotaxime were observed to be resistant in bacteria having AmpC β-lactamases. However, aztreonam and cefepime are usually more active against bacteria possessing class C β-lactamases. Earlier, the encoding gene, ampC was thought to be located exclusively on chromosome but, now an increasing number of ampC genes have been identified on plasmids [Walther-Rasmussen *et al.*, 2002], and mostly acquired by AmpC deficient pathogenic bacteria, which consequently are supplied with new and additional resistance phenotypes. Bush group 2f enzymes (Ambler class D), serine β-lactamases, are able to hydrolyze oxacillin and, hence knows as oxacillinases or OXA β-lactamases. *Acinetobacter baumannii* and *P. aeruginosa* represent the most structurally diverse and rapidly growing group of OXA β-lactamases [Brown *et al.*, 2006; Walther-Rasmussen *et al.*, 2006]. These β-lactamases due to OXA enzyme confer resistance to penicillins, cephalosporins, extended spectrum cephalosporins (OXA-type ESBLs) or carbapenems (OXA-type carbapenemases). OXA enzymes are inhibited by sodium chloride but found to be relatively resistant to clavulanic acid inactivation, [Naas *et al.*, 1998; Poirel *et al.*, 2002].

1.3.2 β-Lactamases mediated resistance to β-lactam antibiotics

TEM-1, TEM-2 and SHV-1 are widespread enzymes that are responsible for resistance to the narrow spectrum cephalosporins, cefamandole and cefoperazone and most of the penicillins. Word "TEM" is derived from Temoniera, name of patient, from whom resistant bacteria were isolated, whereas SHV is abbreviation of sulfhydryl variable that indicate the biochemical properties of this β-lactamase [Heritage *et al.*, 1999]. In epidemiological study, it has been found that plasmid mediated enzymes TEM-1, TEM-2 and SHV-1 most commonly encountered with TEM-1 predominant and responsible for 90% ampicillin resistance in *E. coli* [Baker *et al.*, 1999]. Amount of TEM and SHV enzymes indicates the degree of resistance and it can vary 150 fold among isolates, reflecting gene dosage and promoter efficiency [Reguera *et al.*, 199; Heritage *et al.*, 1999]. Recognition and identification of these enzymes may be made on the basis of biochemical criteria (substrate profiles, kinetic properties and reaction with inhibitors),

physical properties (molecular size and isoelectric point), or by genetic criteria, such as inducibility and location of their genes on plasmids or on the chromosome [Philippon *et al.*, 1989; Jacoby *et al.*, 1994; Livermore & Williams, 1996]. β-lactamases can produce antibiotic resistance through multiple ways. In some bacteria as in *E. coli* (TEM-1 producers), the β-lactamases remain localised in the periplasmic space and can destroy antibiotic molecules when make their way through the outer membrane and as a resultant, high level of resistance occurs within a single bacterial cell [Medeiros *et al.*, 1984; Zhou et al., 1994]. Affinity for the antibiotic (Km), rate of hydrolysis (Vmax), and the amount of β-lactamase are among the most important factors that contribute to the level of antibiotic resistance mediated by a particular β-lactamase in a population of bacteria. [Pitout *et al.*, 998]. In eight decade of last century, extended spectrum cephalosporin resistance due to production of β-lactamases has been observed in Gram negative bacteria. And it was also observed that first extended spectrum β-lactamases was derived from SHV-1 or TEM-1 β-lactamases after mutation. The genes encoding these mutants are present on mobile genetic elements, facilitating their spread in nosocomial pathogens [Heritage *et al.*, 1999].

a. TEM-1 β-lactamases

Most common plasmid encoded β-lactamase, TEM-1, was first discovered in *E.coli* in 1965 [Bryan & Godfrey, 1991; Ho *et al.*, 1998], and has been observed spreading to 20-60% of *Enteriobacteriaceae* isolates with varying frequency in species and location. Its expression is nothing but constitutive with variable concentration among strain. Ampicillin, amoxicillin and ticarcillin were found to be resistant to lesser concentration of TEM-1 while piperacillin, mezlocillin, cephalothin, cefamandole and cefoperazone were observed to be resistant to higher concentration of TEM [Livermore *et al.*, 1993].

b. SHV-1 β-lactamases

SHV-1 is classified in the Bush group 2b enzymes [Sanders & Sanders., 1992] and is encoded by SHV-1 gene, commonly encountered in clinical isolates on self-transmissible

plasmids. It was first described as a chromosomally encoded β-lactamase in *Klebsiella* [Heritage *et al.,* 1999]. It is a narrow spectrum β-lactamase and has activity against penicillin. Production and concentration of enzyme amongst strains is variable more than one hundred fold as it depends on the number of plasmid copies per organism, the degree of gene amplification and the efficiency of the promoter and [Jacoby & Carreras, 1990; Abdel-Rahman & Kearns, 1998; Petrosino *et al.,* 1998].

1.3.3 Extended spectrum β-lactamases (ESBLs)

Extended spectrum β-lactamases (ESBLs) are globular proteins composed of alpha-helices and beta-pleated sheets [Knox *et al.,* 1995]. β-lactamases hydrolyze extended-spectrum cephalosporins with an oxyimino side chain, e.g., CTX-M [http://en.wikipedia.org / wiki / Beta-Lactamase]. Plasmid encoded β-lactamases; TEM-1, TEM-2, and SHV-1 in *Enterobacteriaceae* family confer resistance to penicillin but not to expanded spectrum cephalosporins [Harbak *et al.,* 2007]. ESBLs are derived from genes for TEM-1, TEM-2, or SHV-1 by mutations that alter the amino acid composition around the active site of these β-lactamases. They are classified in group 2be in Bush-Medeiros-Jacob system and class A in Ambler system. It was observed that point mutation in SHV and TEM β-lactamases which cause single amino acid substitution (Asp 104- Lys, Arg164-Ser, Arg164-His, Asp179-Asn, Gly238-Ser and Glu240- Lys) are responsible for the resistance[Philippon & Jacoby, 1989 & Medeiros,1991]. Hence these enzymes are potent weapon of bacteria as they extend the susceptibility of β-lactam antibiotics to hydrolysis. [Harbak *et al.,* 2007; http://en.wikipedia.org/wiki/ Beta-lactamase]. ESBLs are usually capable of hydrolyzing penicillins (e.g. ampicillin and piperacillin), cephalosporin of first, second, third and fourth generation and the monobactam azotreonam but not to cephamycins or carbapenems [Amber *et al.,* 1991]. The distinctive property of ESBLs (e.g. members of TEM and SHV families) of being exploited by β-lactamase inhibitors such as clavulanic acid, tazobactam or sulbactam, is duly exploited in double-disk synergy test meant for ESBL detection [Rossolini *et al.,* 2006]. The ESBLs are frequently plasmid encoded and plasmids responsible for ESBL production frequently carry genes encoding resistance to other drug classes (for example,

aminoglycosides) [Bradford *et al.*, 2001]. Therefore, antibiotic options in the treatment of ESBL-producing organisms are extremely limited. ESBL producing organisms may appear susceptible to some extended-spectrum cephalosporins. However, treatments with such antibiotics have been associated with high failure rates [http://en.wikipedia.org/wiki/Beta-Lactamase]. Carbapenems are the treatment of choice for serious infections due to ESBL producing organisms, yet carbapenem resistant isolates have recently been reported [Rossolini *et al.*, 2006; Bradford *et al.*, 2001].

1.3.4 CTX-M β-lactamases

CTX-M is a recently described family of the extended-spectrum β-lactamases [Tzouvelekis *et al.*, 2000] and they are now considered the most prevalence ESBLs worldwide [Livermore *et al.*, 2007]. These enzymes show potent hydrolytic activity against cefotaxime and hence they are called as CTX-M [Bonnet *et al.*, 2004]. Bush *et al.*, (1993) has reported that they hydrolyze cephalothin better than benzylpenicillin and preferentially hydrolyze cefotaxime over ceftazidime. CTX-M inhibition is observed to a greater extent in Tazobactam than in sulbactam or clavulanate [Bush *et al.*, 1993]. They generally found as plasmid acquisition of β-lactamases genes on chromosome of Kluyvera species. [Humeniuk *et al.*, 2002]. CTX-M type β-lactamases have 40% or less identity with TEM and SHV type ESBLs CTX-M β-lactamases. The number of CTX-M type β-lactamases, which is most frequent type of ESBLs worldwide, is rapidly expanding and more than 40 CTX-M variants are identified in *K.penumonia, E.coli*, typhoidal and non typhoidal *Salmonella, Shigella, Citrobacter freund, Enterobacter* spp., *and Serratia marcescens* [Walther-Rasmussen & Hoiby, 2004; Tzouvelekis *et al.*, 2000; Bradford *et al.*, 1998]. CTX-M enzymes can be sub classified by amino acid sequence similarities and on the basis of phylogenetic studies; acquired CTX-M enzymes may be divided in another five major groups.

Selective remodeling of active sites to accommodate the bulky R1 side chain of extended spectrum cephalosporins is one of the most important observations as revealed in study of the atomic structures of class A ESBLs [Knox *et al.*, 1995]. Amino acid residues Asn104,

Asn132, Ser237, and Asp240 have been identified as the key factors behind the substrate specificity of the CTX-M β-lactamases by several studies that used comparative sequence analyses, modeling, and mutagenesis techniques [Bauernfeind *et al.*, 1996]. Ser 237 has been observed to be involved in the extension of the substrate specificities of TEM and SHV ESBLs to cefotaxime [Knox *et al.*, 1995]. The Ser237Ala substitution in the CTX-M-4 enzyme induces a decrease both in relative hydrolytic activity against cefotaxime and in susceptibility to inhibition by clavulanate [Gazouli *et al.*, 1998]. The acyl intermediate structure of Toho-1 in complex with cefotaxime shows a rotation of the Ser237 side chain, which prevents steric clashes with the methoxyimino group of cefotaxime and which allows the formation of a hydrogen bond with the carboxylate group of cefotaxime [Shimamura *et al.*, 2002]. It has been suggested that this interaction assists in bringing the carbonyl group of the β-lactam ring of cephalosporins to the optimal position in the oxyanion hole for acylation [Shimamura *et al.*, 2002]. The relatively low penicillinase activities of CTX-M enzymes may be caused by Van der Waals contact between residue Ser237 and the methyl group of the thiazolidine ring. Asn104, Asn132, Ser237, and Asp240 residues establish hydrogen bonds with the amide and aminothiazole groups of the acyl-amide-cefotaxime chain. This unusual acyl intermediate of CTX-M enzymes in complex with cefotaxime may therefore be involved in the activities of the oxyimino-cephalosporinases by fixing cefotaxime tightly in the binding site [Shimamura *et al.*, 2002]. The structure of Toho-1 revealed that the omega loop (amino acid positions 161-179) has fewer hydrogen bond interactions with the β3 strand in the vicinities of Asn170 and Asp240 than the restricted-spectrum β-lactamase of *Bacillus licheniformis*, the enzyme most closely related to Toho-1 at the structural level. No hydrogen bond has been observed between the Phe160 residue and Thr181 and Asp157 residues, which both connect the N and C termini of the omega loop in restricted-spectrum β-lactamases [Ibuka *et al.*, 1999]. These structural features may increase the flexibility of the omega loop. The structures of acyl intermediates of the Toho-1 enzyme show a shift of the omega loop to helix H5 [Shimamura *et al.*, 2002] as a result of a complex structural rearrangement in the hydrophobic core in the vicinity of the omega loop (the residues involved in the rearrangement are Cys69, Ser72, Met135, Phe160, and Thr165). This shift narrows the binding site, but the steric contacts of the Pro167 and

Asn170 residues with the aminothiazole ring of cefotaxime are avoided. Mutants with point mutations in common CTX-M enzymes exhibiting improved catalytic efficiencies against ceftazidime have been observed [Knox *et al.*, 1995]. The change in activities of CTX-Ms leading to the evolution of more variants may be due to point mutations present either inside or outside of the active site omega loop (amino acid positions 161 to 179) [Sturenburg *et al.*, 2004]. For example the P167T mutation differentiates CTX-M-23 from CTX-M-1, CTX-M-3 and CTX-M-15. However the CTX-Ms, having identical residues present in the omega loop may still have some difference in their enzymatic activities due to mutations present outside the omega loop. The CTX-M-15, CTX-M-16, and CTX-M-27 enzymes harbor the Asp240Gly substitution. The presence of Lys and Arg residues at position 240 are known to increase the enzymatic activities of the TEM and SHV types ESBLs against ceftazidime [Knox *et al.*,1995]. The Lys and Arg residues are positively charged and can form an electrostatic bond with the carboxylic acid group on oxyimino substituents of ceftazidime [Huletsky *et al.*, 1993; Cantu *et al.*, 1996]. Neutral residue Gly240 is not able to form electrostatic interactions with β-lactams but could favor the accommodation of the oxyimino-ceftazidime side chain [Bonnet *et al.*, 2001; Bonnet *et al.*, 2003].

1.3.5 OXA β-lactamases

The OXA β-lactamases belong to class D (2d) in Ambler classification [Ambler, Coulson *et al.* 1991], mainly occur in *Acinetobacter* and *Pseudomonas* species. The OXA β-lactamases is responsible to attack the oxyimino-cephalosporins. They also have a high hydrolytic activity against oxacillin, methicillin and cloxacillin more than benzylpenicillin and inhibited less efficiently by clavulanate but efficiently with NaCl [Heritier *et al.*, 2005; Poirel and Nordmann, 2006; Walther-Rasmussen and Hoiby, 2006]. At present, more than 180 different variants of OXA enzymes have been identified on the protein level (http://www.lahey.org/studies/). Most of the genes encoding class D oxacillinases have commonly been found on plasmids incorporated as gene cassettes in integrons. For example, OXA-58 has been reported to confer resistance to imipenem through plasmid mediated gene transfer in *A. baumannii* [Zarrilli *et al.*, 2008]. Several

chromosomal encoded oxacillinases have been reported [Heritier, Poirel *et al.*, 2005; Walther- Rasmussen & Hoiby., 2006]. Most OXA-type β-lactamases do not hydrolyse the extended-spectrum cephalosporins and hence they are not regarded as ESBLs. OXA-11 a variant of OXA-10 (previously known as PSE-2), the first extended-spectrum from *P. aeruginosa* 29 ABD is identified in October 1991 from blood cultures of a burn patient in Turkey [Hall, Livermore *et al.*, 1993]. Substitutions of an asparagine for serine at position 73, or an aspartate for glycine at position 157 are probably required for the ESBL phenotype [Bradford *et al.*, 2001]. Most OXA-type ESBLs derive from OXA-10 (OXA-11, OXA-13, OXA- 14, OXA-16, OXA-17 OXA-19 and OXA-28), to a lesser extent from OXA-2 (OXA-15 and OXA-32), and others unrelated to any recognized broad spectrum OXA enzymes (OXA-18 and OXA-45) [Naas *et al.*, 2008]. In 1993, Paton *et. al* identified and described the first OXA β-lactamase ARI-1 (for "*Acinetobacter* resistant to imipenem") as OXA-23 with carbapenemase activity from a strain isolated in 1985 from a patient in the Royal Infirmary of Edinburgh, Scotland. Carbapenems hydrolysis by the class D oxacillinase in *A. baumannii* was classified into four subgroups of eight clusters and they have been designated OXA-23-like; OXA-40-like; OXA-51-like and OXA-58-like [Brown & Amyes., 2006], whereas Walther-Rasmussen and Hoiby (2006) subclassified carbapenem-hydrolysing OXA enzymes into eight distinct branches or subgroups including OXA-23, OXA-24, OXA-48, OXA-50, OXA-51, OXA-55, OXA-58 and OXA-60. OXA-62 is another subgroup carbapenem-hydrolyzing oxacillinase which was identified in *Pandoraea pnomenusa* from cystic fibrosis patients [Schneider *et al.*, 2006]. Poirel *et al* (2005) has observed OXA-51-like enzymes in all tested *A. baumannii* strains.

1.4 Neonatal infections

Immature immune system, particularly of preterm baby is one of the most important causes for neonatal infection [Fanos *et al.*, 2007]. Because antibodies which help and protect the mothers from infections do not cross through the placenta to the fetus until approximately 30 weeks of gestation and also they do not reach up to optimum level at birth time and hence affects the protection provided. Neonatal Infection may be classified

into three main groups: intrauterine, intrapartum, and postnatal infections. These three groups have several factors that increase the risk of neonatal infection.

Intrauterine infections occur when pathogenic organisms cross the placenta into the fetal circulatory system. The organisms, such as cytomegalovirus (CMV), can reside in the amniotic fluid. Other organisms ascend from the vaginal track, infecting the membranes and causing them to rupture. This rupture of membranes can lead to infections of the respiratory and gastrointestinal tract of a newborn. Therefore, poor prenatal care, poor nutrition, recurrent abortions, and substance abuse are identified as most important risk factors for intra uterine neonatal infection. Factors which are responsible for neonatal infection during the birth process or intra-partum period include: prolonged rupture of membranes (>12 to 18 hours), urinary tract infections, preterm labor, prolonged or difficult labor, maternal fever and maternal infections. Most infections during delivery of fetus are related to the infant coming into unavoidable contact with an infected birth canal. The birth canal can host bacteria that an infant's immune system cannot defend against infections in the postnatal period which are more common in those infants who require foreign objects to be introduced into their systems. Items like endo-tracheal tubes or indwelling catheters increase the neonatal infection. This is much commonly observed during resuscitation, or as a result of a nosocomial infection due to improper hand washing. But, the single most important risk factor for infection in the neonate is prematurity [Seo *et al.,* 1992]. However, factors that increase the infant's chance of becoming sick include: low birth weight (Infants weighing less than 1000g are at the highest risk for infections), prematurity, birth asphyxia, meconium staining, and resuscitation. Premature neonates are generally supposed to have higher risk of developing infection due to immature immune system, thinner skin, frequent need for insertion of foreign objects and use of prolonged ventilator in high risk of neonates for chronic lung disease [Elster *et al.,* 2009]. Some life saving drugs like corticosteroids which are essential to help and treat illness of neonates may leave the infant at an increased risk of infection. Pathogen found in cerebrospinal fluid (CSF), urine, and bacterial blood cultures are the main source that confirms infection in a neonate. Some other pathological condition like hypoglycemia, hyperglycemia, metabolic acidosis, thrombocytopenia, or hyperbilirubinemia may be taken as another source and may be

checked in an infant suspected of sepsis. The blood culture and complete blood count (CBC) is the most important tool to recognize the causative organism and infection. Increased or decreased white blood count (WBC), low platelet count and a high I:T ratio indicate the presence of infection [Aulia *et al.,* 2003]. I:T ratio is used an index to show the percentage of immature white blood cells to total white blood cells. Greater I:T ratio, more than 0.2, indicates a "left shift" that means more immature neutrophils than mature neutrophils are circulating in the bloodstream due to acute infective condition. And when the severity of infection declines neutrophils come back to its normal level, a "shift to the right" occurs, indicating that everything is in its normal limit.

1.4.1 *Klebsiella pneumoniae* and neonatal infections

K. pneumoniae, an opportunistic pathogens mainly in immunocompromised and in other high risk patient groups, is responsible for community acquired infections such as primary lobar pneumonia and 8-13% of cases of UTIs. [Murray *et al.,* 1998]. Nosocomial infections and infections of wound or soft tissues are also known to be caused by this opportunistic pathogen. [Vaara & Vaara, 1983; Murray *et al.,* 1998]. Patients with central venous and urinary tract catheters, patients on mechanical ventilation and people with compromised pulmonary function are also at risk of being infected with *K. pneumoniae* [Jarlier *et al.,* 1988; Weiner *et al.,* 1999; Rebuck *et al.,* 2000; Lautenbach *et al.,* 2001]. Presence of cell wall receptor, K antigen, production of long chain -O- antigen and large plasmid are the most important factors which contribute to the virulence of *K. pneumoniae* as cell wall receptors enable the organism to attach to host cells and protect the bacteria from phagocytosis and intracellular killing by polymorph nuclear leukocytes where as K antigen, an extensive polysaccharide capsule, protects the bacterial cell from phagocytosis and directly suppresses the immune response [Mims *et al.,* 1993; Hooton *et al.,* 2000]. Production of long chain-O-antigen polysaccharide in the endotoxin of the outer membrane may contribute to resistance by inhibiting complement-mediated serum killing. And large size of plasmid (>200 kb) encodes aerobactin (a protein involved in iron acquisition and regulation of the mucoid phenotype) and thus becomes a reason for virulence of *K. Penumoniae.*

1.4.2 *E. coli* and neonatal infections

It is worldwide established fact that *E. coli* is the most common cause of gram negative neonatal infections. Mother's genital tract is thought to be primary source of colonization in neonates. The pathogen has a wide spectrum pathological manifestation in human being and can cause respiratory distress, cardiovascular collapse, meningitis, multi-organ failure, and even death. Gentamicin and amikacin are drugs of choice for treatment of *E.coli* mediated infective conditions in neonatal intensive care unit.

1.5 Diabetic foot ulcer/infection

According to World Health Organization diabetic foot is defined as 'the foot of a diabetic patient that has the potential risk of pathologic consequences, including infection, ulceration, and destruction of deep tissues associated with neurologic abnormalities , various degrees of peripheral arterial disease, and metabolic complications of diabetes in the lower limb' [Frykberg *et al.*, 2006]. In India, diabetes is the most alarming health problem and is expected to increase to 57 million populations by 2025 [Abdul *et al.*, 1999]. Diabetic Foot ulcer is the most common single precursor to amputation and has been identified as a component in 85% of lower- extremity amputations [Margolis *et al.*, 2005]. It has also been reported that 15% of diabetic patients would develop a foot ulcer during the course of their disease [Boulton *et al.*, 2004]. Neuropathy, and impaired blood supply in combination with deformities of the feet and the resulting increased pressures on areas of the sole are the most important pathophysiological factors. History of previous foot ulcers and amputation, inadequate footwear, a lack of (or low-quality) podiatry care, poor diabetes management, long duration of diabetes, chronic renal disease, poor visual acuity, psychological factors and behavioral factors, smoking, old age and low social status are also important contributing factors.

Diabetic foot infection is a broader term that includes paronychia, cellulitis, myositis, abscesses, necrotizing fasciitis, septic arthritis, tendonitis, and osteomyelitis in association of diabetes mellitus. But the most common form of diabetic foot infection is diabetic foot ulcer which mainly results from neuropathy, ischemia and other complex

mechanism [Lipsky *et al.*, 2004]. Because, diabetic foot ulcer that breaches the protective layer of skin and exposes the underlying soft tissue, provides a better media for bacterial colonization. Due to this bacterial colonization and ischemic condition, the wound may further progress to become actively infected and involve deeper tissue. However, several other pathophysiological factors increase the risk and severity of foot infections [Schubert & Heesemann., 1995]. But, abnormal cellular, inflammatory and immunological response to bacterial toxins in diabetes causes increased vulnerability to infection and delayed wound healing process that ultimately complicate the ulceration [Yan *et al.*, 2003; Delamaire *et al.*, 1997]. Increased Blood glucose level also increases the rate of infection in diabetic foot ulcer. Arteriovenous communication and impaired intermediary tissue metabolism in diabetic patients also predispose them to infection and then compound the problem by potentiating infection once a pathogen has been introduced.

Etiology Among the various causative organism isolated from infected foot ulcer, staphylococcus has been predominantly observed [Xu *et al.*, 2007; Yates *et al.*, 2009]. *Pseudomonas aeruginosa* [Martínez-Gómez *et al.*, 2009], *E. coli* [Varaiya *et al.*, 2008], *Klebsiella* species [Goldstein *et al.*, 2008], *Enterococcus* species [Martínez-Gómez *et al.*, 2009], and *Proteus* species [Raja *et al.*, 2007] have also been observed and reported in various diabetic foot ulcer cases. But the classical signs and symptoms of these pathogen mediated infection remain hidden in two third patients of infective diabetic foot ulcer probably due to neuropathy, alteration in microcirculation and leukocyte abnormalities. Extensive foot infection, and increased blood glucose level are remain the sole presenting sign.

Wagner Classification System Various classification systems are made to define and determine the severity of diabetic foot ulcer. Most common and widely used classification system is the Wagner System [Wagner *et al.*, 1981]. This system is basically anatomical with gradations of superficial ulcer, deep ulcer, abscess osteitis, gangrene of the fore foot, and gangrene of the entire foot. Only grade 3 addresses the problem of infection. In this system, foot lesions are divided into different grades starting from grade 0 to grade 5. Grade 0 includes high risk foot but no active lesion and grade 5 includes gangrene of entire foot.

Diabetic foot infection and antibiotic regime As a general rule, control of infection is the first priority in the management of diabetic foot problem. Even minor infected foot lesions in the diabetics should always be taken seriously and treated early with suitable antibiotics. Selection of the antibiotic regimen mainly depends on the route of drug administration, the spectrum of microorganisms to be covered, the specific drugs to administer, and the duration of treatment. For severe infections and for more-extensive, chronic moderate infections, it is safest to commence therapy with broad-spectrum agents. These should have activity against gram-positive cocci (including MRSA in locations where this pathogen is common), as well as gram-negative and obligate anaerobic organisms. Clinical trials suggest that fluoroquinolones, cephalosporins, β-lactam inhibitor penicillins, and carbapenems are effective. These suggested agents are derived from available published clinical trials and are not meant to be inclusive of all potentially reasonable regimens. Similar agents could be used, depending on various clinical, microbiological, epidemiological, and financial considerations such as imipenem which was reported equally effective against Gram-positive and negative diabetic foot isolates and vancomycin against gram-positive isolates [Raja *et al.*, 2007].

1.6 Molecular characterization/Typing of clinical isolates

Different methods are being used to type bacterial isolates, which include restriction endonuclease assay (REA) [Savio *et al.*, 1994; Brown & Levett, 1997] , pulsed field gel electrophoresis (PFGE) [Herrmann *et al.*,1991; Herrmann *et al.*, 1992], restriction fragment length polymorphism (RFLP) [Zuerner *et al.*, 1993], arbitrarily primed PCR [Perolat *et al.*, 1994], Variable Number of Tandem Repeats (VNTR) analysis [Majed *et al.*, 2005] and fluorescent amplified fragment length polymorphism (FAFLP) [Vijayachari *et al.*, 2004]. These methods are considered to be valuable tools for distinguishing the bacterial strains or clones and as an integral part of clinical as well as microbiological research. These methods are highly discriminatory and have added greatly to our knowledge of the epidemiology of many pathogenic bacteria in outbreak settings, such as methicillin-resistant *Staphylococcus aureus* (MRSA) [de Lencastre *et*

al., 1996; Roberts *et al.*, 1998; Macfarlane *et al.*,1999] and vancomycin-resistant enterococcal infections in hospitals [Morrison *et al.*, 1999] and outbreaks of *E. coli* O157:H7 food poisoning [Gouveia *et al.*, 1998; Besser *et al.*, 1999] and meningococcal meningitis [Jelfs *et al.*, 1998] within the community.

1.6.1 Multilocus sequence typing (MLST):

MLST, a novel molecular typing method based on the principles of multilocus enzyme electrophoresis (MLEE) [Selander *et al.*, 1986] has been developed and used in study of many bacterial species like *Neisseria meningitides* [Maiden *et al.*, 1998], *Streptococcus pneumoniae* [Enright & Spratt., 1998], *Streptococcus pyogenes* [Enright *et al.*, 2001], *Staphylococcus aureus* [Enright *et al.*, 2000], *Yersinia* species [Kotetishvili *et al.*, 2005.], *Campylobacter jejuni* [Dingle *et al.*, 2001] and *Helicobacter pylori* [Devi *et al.*, 2006].
It is a simple nucleotide sequence-based PCR technique for characterizing the genetic relationships among bacterial isolates, which makes use of automated DNA sequencers to assign and characterize the alleles present in each locus. Nucleotide sequencing explores all of the variation at a locus. Number of alleles assigned per locus is much higher than in MLEE and the sequences of 450-500-bp internal fragments of only seven housekeeping genes are determined for each isolate. This length of DNA fragment can be sequenced accurately on both strands using a single pair of primers and, in most bacterial pathogens; it provides sufficient variation to identify many different alleles within the population.

Different sequences, for each gene, are assigned as alleles and the alleles at the seven loci provide an allelic profile, which unambiguously shows the sequence type (ST) of each isolate. Sequences, if are different at even a single nucleotide, are assigned as different alleles and no consideration will be given to the number of nucleotide differences between alleles, as it cannot be distinguished whether differences at multiple nucleotide sites are a result of multiple point mutations or a single recombinational exchange. Allele at each of the seven loci provides the ability to differentiate billions of different allelic profiles. Strain with the frequent allele at each locus occur by chance approximately once in 10 000 isolates. It is rare that two unrelated isolates will have the same allelic profile [Spratt *et al.*, 1999].

MLST is highly discriminatory generating unambiguous sequence data in a low to high-throughput scale, and the accumulation of nucleotide changes in housekeeping genes is a relatively slow process and stable for longer duration [Enright *et al.*, 1999]. Hence, the allelic profile of a bacterial isolate may be used for global epidemiology as highly robust markers of ancient and modern ancestry. A dendrogram, to find out the relatedness of isolates, may be prepared using the matrix of pair-wise differences between their allelic profiles. The dendrogram is display of isolates having identical or very similar allelic profiles and that can be assumed to be derived from a common ancestor. Difference at more than three loci out of seven loci in bacterial isolates cannot provide any relationship among them and should not be considered for the inference of their phylogeny [Spratt *et al.*, 1999; Spratt & Maiden., 1999]. It also provides computerized data that allow multi-user international databases available. The sequencing of multiple loci provides a balance between technical feasibility and resolution. Recent studies showed that MLST is more appropriate for strain phylogeny and large scale epidemiology. Analysis of nosocomial isolates showed that MLST can discriminate among epidemiologically unrelated isolates [Diancourt *et al.*, 2005; Vimont *et al.*, 2008].

1.6.2 Replicon typing/Incompatibility

As mentioned above, plasmids are extra-chromosomal circular fragments of DNA and replicate autonomously in a host cell. They are usually seen in all bacterial species in variable size ranging from a few to more than several hundred kilobase pairs. [Waters *et al.*, 1999]. Plasmids are thought to contain a backbone of core genes that remains relatively stable for long evolutionary periods and simultaneously increases the genetic diversity of bacteria by acquisition and loss of genes as they horizontally exchange among bacterial species by conjugation or mobilization [Francia *et al.*, 2004]. Beside the core genes that are responsible for initiation and control of replication, some accessory genes that may be useful to their bacterial host such as antimicrobial resistance or virulence genes, are also found here [Amabile- Cuevas and Chicurel, 1992; Bergstrom *et al.*, 2000; Thomas *et al.*,1973]. The identification and characterization of the core genes of a plasmid species has a special relevance in the study of its epidemiology and modes

of transmission that will explore the main routes of that genes, for example antibiotic resistance genes, use to travel from environmental reservoirs to human pathogens. Because, worldwide dissemination of plasmid mediated multiple antibiotics resistance and virulence traits is an increasing threat for the treatment of many bacterial diseases. To follow the dissemination of virulence and antibiotic resistance genes, identification and categorization of the causative plasmids and following of their path from reservoirs to pathogens is necessary. Novick (1987) has developed the incompatibility grouping for classification and categorization of plasmid. He introduced, by conjugation or transformation, a plasmid of an "unknown" Inc group into a strain carrying a plasmid of a known Inc group. The incoming plasmid of unknown Inc group will be assigned to its same Inc group if the resident plasmid is eliminated in progeny [Datta & Hedges., 1971]. So, on the basis of this expression, plasmids with the same replication control and plasmids with different replication controls are termed as incompatible and compatible plasmid and two plasmids of same Inc group cannot be propagated in the same cell line [Datta and Hughes, 1983; Couturier *et al.*, 1988].

Incompatibility method has been frequently used to classify and to identify the plasmid, to trace the diffusion of plasmids conferring antimicrobial resistance, and also to follow the evolution and spread of emerging plasmids [Anderson *et* al., 1977]. An another method based on hybridization with 19 DNA probes that recognize different basic replicons, for the identification of the major replicons of plasmids circulating among the *Enterobacteriaceae*, was developed by Couturier *et al* (1988). But this conjugation and hybridization based methodologies cannot be easily applied to a large number of strains and their application has been limited by the laborious and time consuming work. For classification and categorization of plasmids, now a days PCR-based replicon typing is the method of choice in clinically relevant bacteria. It was observed that the PCR-based method can be applied directly on collections of strains to track the spread of plasmids conferring drug resistance, monitor the circulation of plasmids within strains from different environments and to follow the horizontal transmission of antimicrobial resistance genes among the *Enterobacteriaceae* [Pezzella *et al.*, 2004].

1. 7 Objectives of the study

In the light of above background, present study was initiated with the following objectives:

1. To find the prevalence and susceptibility pattern of plasmid mediated ESBL-producing *E. coli*, *Klebsiella pneumoniae* and *pseudomonas* species in the Intensive care unit (ICU) of Aligarh, hospital, India.

2. To identify the risk factors associated with the transmission of bla_{CTX-M}, bla_{TEM} and bla_{SHV} among the ESBL-producing *K. pneumoniae* strains of the NICU.

3. To characterize the mode of transmission of bla_{CTX-M}, bla_{TEM} and bla_{SHV} among the ESBL-producing *K. pneumoniae* strains of the NICU.

4. To understand the Molecular mechanism of inhibitor resistant ESBL producing *K. pneumonia* isolated from NICU.

5. To screen the metallo-β-lactamase (MBL) producing isolates in diabetic foot ulcer patients of endocrinology ward and ICU at the JNM College and hospital, Aligarh.

Chapter-2

Material and Methods

2.1 Materials

Materials used during the study are given below:

2.1.1 Chemicals, reagents and kits used:

Agarose	SRL, India
Bromophenol blue	Hi-Media, India
$CaCl_2$	SRL, India
Chloroform	SRL, India
Cotton swab	Hi-Media, India
DNA ladder (100 bp)	Genei-Bangalore, India
DNA ladder (1kb)	Genei-Bangalore, India
DNA ladder (10 kb)	Fermentas, Canada
EDTA	SRL, India
Ethanol	E. Merck, India
Ethidium bromide	Sigma, USA
Genomic DNA isolation kit	Qiagen, USA
Glacial acetic acid	E. Merck, India
Glycerol	SRL, India
Gel extraction Kit	Qiagen, USA
HiComb MIC Test strips	Hi-Media, India
Hydrochloric acid	SRL, India
Isoamyl alcohol	SRL, India
Lysozyme	Sigma, USA
$MgCl_2$	SRL, India
NaOH	SRL, India
Plasmid DNA isolation kit	Qiagen, USA
PCR master-mix	Genei-Bangalore, India
Proteinase K	Sigma, USA
RNase	Sigma, USA
SDS	Hi-Media, India
Sodium acetate	SRL, India

Tris-saturated Phenol	Genei-Bangalore, India
Tris-HCl	E. Merck, India
Xylene cyanol	Bio-Rad, USA

2.1.2 Culture media:

Following culture media used for the isolation and identification of the organism was purchased from Hi-Media, Pvt. Ltd. Mumbai, India.

Agar agar
Brain-Heart Infusion Broth.
EMB-Broth
Luria-Bertani broth
Mac-Conkey agar
Mueller-Hinton agar
Mueller-Hinton broth
Nutrient agar
Nutrient broth
Selective media of Hi-crome series (Hi-media, Mumbai, India), which are highly specific for single target microorganism each (e.g. Hi-crome *Klebsiella* selective agar, Hi-crome *E.coli* selective agar)

2.1.3 Antibiotics:

All of the following antibiotic discs were purchased from Hi-Media, Pvt. Ltd, Mumbai, India.

Amikacin	Ak	30 µg
Amoxyclav	Ac	20/10 µg
Ampicillin/Sulbactam	As	10/10 µg
Amoxicillin/Sulbactam	Ams	10/10 µg
Aztreonam	Ao	30 µg

Cefaclor	Cj	30 µg
Cefalothin	Ch	30 µg
Cefazolin	Cz	30 µg
Cefepime	Cpm	30 µg
Cefepime/ Tazobactam	Cpt	30/10 µg
Cefixime/Clavulanic acid	Cmc	30/10 µg
Cefotaxim	Ce	30 µg
Cefoxitin	Cn	30 µg
Ceftazidim	Ca	30 µg
Ceftazidim/Clavulanic acid	Cac	30/10 µg
Ceftriaxone	Ci	30 µg
Ceftriaxone/Sulbactam	Cis	30/10 µg
Ceftriaxone/Tazobactam	Cit	30/10 µg
Cefuroxim	Cu	30 µg
Chloramphenicol	C	30 µg
Ciprofloxacin	Cf	5 µg
Co-trimoxazole	Co	1.25/23.75 µg
Ertapenem	Ert	30 µg
Erythromycin	E	15 µg
Gatifloxacin	Gf	5 µg
Gentamicin	G	10 µg
Imipenem	I	10 µg
Meropenem	Mr	30 µg
Methicillin	M	5 µg
Norfloxacin	Nx	30 µg
Oxacillin	Ox	30 µg
Piperacillin	Pc	100 µg
Tetracyclin	T	30 µg
Tobramycin	Tb	30 µg

2.2 Methods

2.2.1 Sample collection and identification of clinical isolates

Sample of clinical isolates were collected from neonatal intensive care unit (NICU) and intensive care unit (ICU) of JNMC hospital, Aligarh, located in north India, which is a tertiary care hospital. Clinical samples were collected from different sources (eyelid, body surface, nose, pharynx, rectum and instruments etc.). Each patient admitted to the ward was weekly examined. Information and history regarding the illness of patients and neonates was obtained from patients, parents and consultant of the ICU and NICU. Clearance was obtained from the institute's ethical committee held on 6 July, 2009. The significant pathogens were identified by standard biochemical procedures [Baron *et al.*, 1994]. Strains of ESBL producing *E.coli*, *K. pneumoniae* and *Pseudomonas* isolated from surveillance swabs and from clinical samples of the patients and neonates were included in the study. All the specimens were identified for the presence of *K. pneumoniae*, *E.coli* and *Pseudomonas* using HiCrome *Klebsiella, E.coli* and *Pseudomonas* Selective Agar Base identification kit (Himedia, Mumbai, India) and final confirmation of bacterial identities was done by 16S rDNA sequencing using the universal primer 27F and 1525R (Table 2.1).

Table 2.1 Primers used in this study

S. No	Primer name	Oligonucleotide Primer sequence (5'-3')		Amplicon size(bp)	Reference/Accession No
		Forward	Reverse		
1.	CTX-M	ATGTGCAGYACCAGTAARGT	TGGGTRAARTARGTSACCAGA	604	Pagani et al., 2003.
2.	TEM	ATAAAATTCTTGAAGACGAAA	GACAGTTACCAATGCTTAATC	1090	AB103506
3.	SHV	CACTCAAGGATGTATTGTG	TTAGCGTTGCCAGTGCTCG	234	AY223863
4.	5'CS,3'CS	GGCATCCAAGCAGCAAG	AAGCAGACTTGACCTGA	variable	M73819
5.	Int1	CTACCTCTCACTAGTGAGGGGCGG	GGGCAGCAGCGAAGTCGAGGC	845	U12338
6.	sul1	ATGGTGACGGTGTTCGGCAT	CTAGGCATGATCTAACCCTC	840	Galimand et al.,2003.
7.	ERIC	ATGTAAGCTCCTGGGGATTCAC	AAGTAAGTGACTGGGGTGAGCG	variable	Versalocic et al.,1991.
8.	OXA-1	TCAACTTTCAAGATCGCA	GTGTGTTTAGAATGGTGA	440	Guessennd et al.,2008.,
9.	KPC	CAGCTCATTCAAGGGCTTTC	AGTCATTTGCCGTGCCATAC	533	Sabine et al.,2009.
10.	NDM-1	GGTTTGGCGATCTGGTTTTC	CGGAATGGCTCATCACGATC	601	Nordmann et al.,2011.
11.	ArmA	ATTTTAGATTTTGGTTGTGGC	ATCTCAGCTCTATCAATATCG	846	Galimand et al.,2003.
12.	RmtA	CTA GCG TCC ATC CTT TCC TC	TTT GCT TCC ATG CCC TTG CC	756	Park et al.,2006.
13.	RmtB	ACTTTTACAATCCCTCAATAC	AAGTATATAAGTTCTGTTCCG	769	Doi et al.,2004.
14.	VIM	GTTTGGTCGCATATCGCAAC	AATGCGCAGCACCAGGATAG		Poirel et al.,2011b
15.	ISAba125	TGTATATTTCTGTGACCCAC	ACACCATTAGAGAAATTTGC		Poirel et al.,2011c
16.	bleo	GGCGATGACAGCATCATCCG			-do-
17.	HI1	GGAGCGATGGATTACTTCAGTAC	TGCCGTTTCACCTCGTGAGTA	471	AF250878
18.	HI2	TTTCTCCTGAGTCACCTGTTAACAC	GGCTCACTACCGTTGTCATCCT	644	BX664015
19.	I1	CGAAAGCCGGACGGCAGAA	TCGTCGTTCCGCCAAGTTCGT	139	M20413
20.	X	AACCTTAGAGGCTATTTAAGTTGCTGAT	TGAGAGTCAATTTTTATCTCATGTTTTAGC	376	Y00768
21.	L/M	GGATGAAAACTATCAGCATCTGAAG	CTGCAGGGGCGATTCTTTAGG	785	U27345
22.	N	GTCTAACGAGCTTACCGAAG	GTTTCAACTCTGCCAAGTTC	559	NC_003292
23.	FIA	CCATGCTGGTTCTAGAGAAGGTG	GTATATCCTTACTGGCTTCCGCAG	462	J01724
24.	FIB	GGAGTTCTGACACACGATTTTCTG	CTCCCGTCGCTTCAGGGCATT	702	M26308
25.	W	CCTAAGAACAACAAAGCCCCCG	GGTGCGCGGCATAGAACCGT	242	U12441
26.	Y	AATTCAAACAACACTGTGCAGCCTG	GCGAGAATGGACGATTACAAAACTTT	765	KO2389
27.	P	CTATGGCCCTGCAAACGCGCCAGAAA	TCACGCGCCAGGGCGCAGCC	534	M20134
28.	FIC	GTGAACTGGCAGATGAGGAAGG	TTCTCCTCGTCGCCAAACTAGAT	262	AH003523
29.	A/C	GAGAACCAAAGACAAAGACCTGGA	ACGACAAACCTGAATTGCCTCCTT	485	X73674
30.	T	TTGGCCTGTTTGTGCCTAAACCAT	CGTTGATTACACTTAGCTTTGGAC	750	K00053
31.	FII	CTGTCGTAAGCTGATGGC	CTCTGCCACAAACTTCAGC	270	AE006471
32.	F	TGATCGTTTAAGGAATTTTG	GAAGATCAGTCACACCATCC	270	AY234375
33.	K	GCGGTCCGGAAAGCCAGAAAAC	TCTTTCACGAGCCCGCCAAA	160	M93063
34.	B/O	GCGGTCCGGAAAGCCAGAAAAC	TCTGCGTTCCGCCAAGTTCGA	159	M28718
35.	rpo	GGC GAA ATG GCW GAG AAC CA	GAG TCT TCG AAG TTG TAA CC	501	Diancourt et al,2005.
36.	gap	TGA AAT ATG ACT CCA CTC ACG G	CTT CAG AAG CGG CTT TGA TGG CTT	450	-do-
37.	mdh	CCC AAC TCG CTT CAG GTT CAG	CCG TTT TTC CCC AGC AGC AG	477	-do-
38.	Pgi2	GAG AAA AAC CTG CCT GTA CTG CTG GC	CGC GCC ACG CTT TAT AGC GGT TAA T	432	-do-
39.	PhoE	ACC TAC CGC AAC ACC GAC TTC TTC GG	TGA TCA GAA CTG GTA GGT GAT	420	-do-
40.	InfB	CTC GCT GCT GGA CTA TAT TCG	CGC TTT CAG CTC AAG AAC TTC	318	-do-
41.	TonB	CTT TAT ACC TCG GTA CAT CAG GTT	ATT CGC CGG CTG RGC RGA GAG	414	-do-
42.	27f/1525	GAGTTTGATCCTGGCTCAG	AGAAAGGAGGTGATCC	1500	Allen et al., 2007.

2.2.2 Antimicrobial susceptibility tests, ESBL, MBL detection and determinations of MICs of ESBL producing strains

The antimicrobial susceptibility was determined by the standard disc diffusion method using Mueller Hinton agar as per the Clinical and Laboratory Standards Institute (CLSI, 2011) guidelines. The antibiotic discs used were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. The ESBL phenotypic confirmatory test with ceftazidime, cephotaxime, ceftriaxone and cefixime was performed for all the probable isolates by disk diffusion method on Mueller-Hinton agar plates with/without 10 mg of amoxyclav. A ≥5mm increase in diameter of inhibition zone with third generation cephalosporins was observed, when tested in combination with amoxyclav, showing ESBL production.

MBL production was detected using combined disc diffusion test by using two imipenem, two meropenem and two ertapenem discs (10μg) with and without 0.1 M ethylene diamine tetra acetic acid (EDTA). One containing 292 μg (10 μl of 0.1M) anhydrous EDTA, which were placed 25 mm apart (center to center) on Mueller Hinton agar plate. An increase in zone diameter of ≥4 mm around the imipenem + EDTA, meropenem + EDTA and ertapenem + EDTA disk compared to that of the imipemen, meropenem and ertapenem disk alone was considered positive for an MBL [Khan *et al.*, 2012]. All ESBL producing strains were also subjected to undergo minimal inhibitory concentrations (MICs) determination using two fold agar dilution susceptibility methods according to updated CLSI guidelines (CLSI, 2011). Appropriate dilutions of β-lactam antibiotic solutions were prepared according to the report of international collaborative study in which one part of the antimicrobial solution was added to nine parts of liquid Muller-Hinton agar. The MIC values were compared with the break points recommended by CLSI. *E. coli* ATCC25922 was used as ESBL negative and *K. pneumoniae* 700603 was used as ESBL positive reference strain.

2.2.3 Statistical analysis

Student t-test and Fishers test for univariate analysis were performed to explore the potential risk factors for ESBL and non ESBL producer strains. Odds ratio (OR) and 95%

confidence intervals (CI) 95% were also performed. Normally distributed continuous variable was analyzed using student t-test. The resistance rate was calculated as the number of resistant strains divided by the total number of strains. The difference was considered statistically significant if p-values were ≤ 0.05. All p-values were two tailed, statistical analysis were performed by Statistical Package for the Social Sciences (SPSS Inc. Chicago, USA) version 16.0.

2.2.4 Resistance transfer experiments

To determine the location of the genes on the chromosome or on a plasmid, conjugation was done by using selected resistant strains as donors and *Escherichia coli* J-53 (EJ-53) (resistant to 250 µg of sodium azide), as selective recipient in all conjugation experiments. Multi-drug resistant donor strains and recipient strain EJ-53 were inoculated into nutrient broth separately and incubated at 37°C until the cells reached logarithmic growth phase. 200 µl of recipient and 50µl of donor were then added to 5 ml of fresh nutrient broth and incubated overnight at 37°C. A loop-ful of the mixture was then spread on the surface of MacConkey agar containing ampicillin (25µg/ml) plus sodium azide (250µg/ml) as selective antibiotics. The plates were incubated overnight at 37°C. Colonies of EJ-53 that grew on the sodium azide /ampicillin selective plates and again on subculture on ampicillin plates were regarded as trans-conjugants, which were further characterized for the presence of resistant markers.

2.2.5 Extraction and preparation of plasmid DNA

Plasmid DNAs were extracted by QIAprep Spin Miniprep Kit (Qiagen, USA). One colony of each strain was inoculated in 5 ml of LB (Luria-Bertani broth) containing a suitable concentration of antibiotic for selection, for 18 hours at 37 °C and centrifuged at 8,000 rpm for 15 minutes at 4 °C to pellet the cells. The pellets were re-suspended in 250 µl of Buffer P1 (50 mM TRIS-HCl, 10 mM EDTA, pH 8.0 containing 100 µg/ml RNase A). 250 µl of Buffer P2 [200 mM NaOH, 1% SDS (w/v)] was added to the samples, mixed thoroughly by vigorously inverting 4-6 times and incubated at room temperature

for 5 minutes. Then 350 µl of chilled Buffer N3 (3.0 M potassium acetate, pH 5.5) was added to the cell lysates, mixed thoroughly by vigorously inverting 4-6 times and incubated on ice for 15 minutes. The samples were centrifuged for 10 minutes at 13,000 rpm at 4 °C. The supernatants containing the plasmids were applied to the QIAprep spin columns. The columns were washed twice with 0.75 ml of Buffer PE [70% ethanol]. The flow-through was discarded, and centrifugation was done for an additional 1 min to remove residual wash buffer. The plasmid DNAs were eluted with 50 µl of Buffer EB [10 mM Tris-Cl, pH 8.5].

2.2.6 Extraction and preparation of total genomic DNA

Total DNA from strains containing studied plasmids were extracted by QIAamp DNA Mini Kit (Qiagen, USA), by the following procedure: one colony from each strain was inoculated in 5 ml of LB (Luria-Bertani broth) containing a suitable concentration of antibiotic for selection, for 18 hours at 37 °C. 1 ml of overnight cultures were transferred in a 1.5 ml micro-centrifuge tube and centrifuged at 7500 rpm for 10 minutes to pellet the cells. To lyse the cells, the pellets were re-suspended in 180 µl of Buffer ATL, 20 µl of proteinase K was added, mixed by vortexing, and incubated at 56 °C until the cells were completely lysed (lysis is usually complete in 1-3 hours). Vortexing was done occasionally during incubation to disperse the sample. 4 µl of RNase Solution (100 mg/ml) was added to cell lysates, mixed by pulse vortexing for 15s, and incubated at 37 °C for 2 minutes. 200 µl Buffer was added to the RNase-treated cell lysates, vigorous vortexing done for 15s and incubation done at 70 °C for 10 minutes. Then, 200µl ethanol (96-100%) was added to the sample, and mixed by pulse vortexing. This mixture was then applied to the QIAamp Mini spin column with 2 ml collection tube and centrifugation done at 8000 rpm for 5 minutes. The QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. 500 µl Buffer AW1 was added and centrifugation performed at 8000 rpm for 2 minutes. The columns were washed again with 500 µl Buffer AW2 and centrifuged at 8000 rpm for 3 minutes. The flow-through was discarded, and centrifugation done for an additional

1 min to remove residual wash buffer. The genomic DNAs were eluted with 200 µl of Buffer AE [10 mM Tris-Cl, pH 8.5].

2.2.7 PCR amplification and molecular typing

Analysis of genetic relatedness of the ESBL-producers was done by the standard method of Versalovic *et al* (1991) using the enterobacterial repetitive intergenic consensus (ERIC)-PCR. PCR amplification was used to find out the bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{OXA-1} bla_{KPC}, bla_{NDM-1}, ArmA and RmtA genes in the isolates which were positive for ESBL synergy test. Presence of genes in selected strains was confirmed by genomic and plasmid DNA screening. Plasmids were extracted by the Qiagen miniprep kit (Qiagen USA). Integrons screening was also performed for the same isolates as described previously [Guerra et al., 2000; Galimand *et al.*, 2003]. Briefly, standard PCRs were carried out in 50 µL volumes containing 5 µL of 10 x PCR buffer (100 mM Tris-HCl (pH 8.8), 500 mM KCl, 15 mM $MgCl_2$, 1% Triton X-100), 5 µL of 10 x deoxynucleotide triphosphate mix (2 mM each dATP, dCTP, dGTP and dTTP) (Sigma,USA.), 2 µL of each primer stock solution (25 pmol/µL), 35 µL sterile distilled water and 1 µL Taq-polymerase (1.5 U/µL diluted solution) (Sigma,USA), and 2-5 µL (100ng-1µg) of template DNA. Amplification was performed in a thermocycler (Applied Biosystem, Veriti, 96-well thermal cycler 9902, Connecticut, USA). The amplification conditions used have also been reported previously [Pagani *et al.*, 2003; Doi *et al.*, 2004, Lee *et al.*, 2006] and the relevant sets of PCR primers are listed in table 2.1. Briefly, PCR conditions used for amplification of bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{OXA-1}, bla_{KPC}, bla_{NDM-1}, *ArmA*, *RmtA*, integrons, 16S rDNA as well as for ERIC-PCR consisted of the following steps: [1] Initial denaturation step of 10 min (95°C) [2] 35 cycles consisting of (i) Denaturation step of 1 min (95°C) (ii) Annealing step of 1 min (50°C for bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{OXA-1}, bla_{KPC}, bla_{NDM-1} and 16S rDNA; 55°C for ArmA, RmtA integrons and ERIC typing) (iii) Extension step of 3 min (72°C) [3] Final extension step of 10 min (72°C) [4] Storage at 4°C.

2.2.8 Agarose gel electrophoresis, staining and visualization of gel

Plasmid DNA, Genomic DNA or PCR products (25 µl) were mixed with 2-8 µl of 10 x TAE buffer and 1-4 µl of bromophenol blue (0.25%). The mixtures were applied to 1-1.5% agarose gels (Sigma, USA) and products separated by electrophoresis for 2-4 h at 90 V using 1 x TAE running buffer (4.84 g/L Tris, 0.37 g/L EDTA, pH 8). To stain the gel after a run, the gel was slid off the platform into a trough containing 0.5µg/ml ethidium bromide solution in 1 x TAE buffer and was let to be stained for 20-30 minutes. The gel was destained in distilled water for 1-2 hours. The DNA samples were visualized by placing gel on a UV transilluminator (254-360 nm) and photographed using gel documentation (Bio-Rad Laboratories, USA). A 100 bp DNA ladder, 1kb DNA ladder and DNA mass ruler (Fermentas, Canada) were included in this study.

2.2.9 Extraction of DNA from agarose gel

PCR products (100 µl) were separated by agarose gel electrophoresis on 1% gels and bands of predicted sizes were cut from the gels with minimum exposure to UV illumination. The DNA was extracted from the gel slices using a DNA extraction kit (Qiagen, USA) by incubation in sodium perchlorate (for extracting fragments <500 bp) or sodium iodide (for fragments >500 bp) at 55°C for 5 min. Resin (15 µl) was then added and the samples were incubated at room temperature for 1 min. After centrifugation at 16000 x g for 30 sec, the pellet was re-suspended in 1 ml washing solution, followed by a 1:1 dilution with 99% ethanol. The sample was again centrifuged at top speed for 30 s and the supernatant was discarded. The pellet was incubated at 55°C (tube with open lid) until all the alcohol had evaporated. Sterile distilled H_2O (10 µl) was added to the pellet after 1 min incubation at room temperature followed by centrifugation for 30s then the supernatant containing the DNA was collected in fresh tube. The final step was repeated to yield approximately 20 µl of DNA sample.

2.2.10 Sequencing of genes

Sequencing of the genes was performed using the same primers which were used for PCR amplifications (Table 2.1). DNA sequences were performed on an ABI 3130 Genetic Analyzer (Applied Biosystems).

2.2.11 PCR-based replicon typing/Incompatibility

Total DNA was obtained from non transconjugants and was subjected to PCR based replicon typing for the identification of major plasmid incompatibility groups among *Enterobacteriaceae*. The plasmid DNA from the non transconjugants were amplified by five multiplex and three simplex PCRs using 18 pairs of primers listed in table 2.1 that recognized Inc replicons FIA, FIB, FIC, HI1, HI2, I1-I, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA [Carattoli *et al.*, 2005].

2.2.12 Multilocus sequence typing (MLST)

MLST is used for the identification and classification of *K. pneumoniae* clinical isolates. The candidate loci sequences were obtained from the strains *rpoB* (beta-subunit of RNA polymerase), *gapA* (glyceraldehyde 3-phosphate dehydrogenase), *mdh* (malate dehydrogenase), *pgi* (phosphoglucose isomerase), *phoE* (phosphorine E), *infB* (translation initiation factor 2) and *tonB* (periplasmic energy transducer) and subjected to MLST. The PCR amplifications of the different MLST target genes were performed using 1.5 mM $MgCl_2$, 200 mM of dNTP's, 25–50 ng template DNA and the relevant sets of PCR primers are listed in table 2.1 using thermo cycler (Applied Biosystem, Veriti, 96-well thermal cycler 9902, Connecticut, USA). Amplification parameters included an initial denaturation at 95°C for 2 min followed by 35 cycles of amplification comprising of de-naturation (95°C for 1 min), annealing (temperature of 50°C for all genes except for *gap*A (60°C) and *ton*B (45°C) and primer extension steps 72°C for 2 min and a final extension of 5 min at 72°C. All the amplified fragments were checked on 1.5% or 2% agarose gel with ethidium bromide staining and the amplicons were sequenced in both the directions using ABI 3130 genetic analyzer sequencers (Applied Bio-systems).

The analysis of sequences obtained for each gene fragment was performed using MLST analysis website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/kpneumoniae.html) For each fragment, the sequences obtained from the clinical strains were compared and allele numbers were assigned to each unique sequence. Each isolate was defined by the combination of numbers corresponding to the alleles at the loci analyzed, which is an allele profile or sequence type (ST). Sequences different even at a single nucleotide site were considered distinct alleles.

Chapter 3

Etiology and susceptibility pattern of plasmid mediated ESBL producers among E.coli, Klebsiella Spp and Pseudomonas strains isolated from intensive care unit (ICU)

3.1 Introduction

Enterobacteriaceae have become one of the most important causes of hospital and community acquired infections [Cantón *et al.*, 2008]. Since 2000, most countries in Europe have experienced a rapid increase resistance in Enterobacteriaceae harboring extended spectrum β-lactamases (ESBLs) [Coque *et al.*, 2008]. This increase in resistance can be largely explained by the dissemination of *E. coli*, *K. pneumoniae* and *pseudomonas* carrying β-lactamases [Livermore *et al.*, 2007]. ESBLs are the bacterial enzymes that impart resistance against advanced generation cephalosporins. There is a widespread occurrence of ESBLs, particularly in hospital environment [Paterson *et al.*, 2005; Pfaller *et al.*, 2006].

ESBLs are dangerous because they are plasmid associated and there can be cross species dissemination of these plasmids. Moreover, these plasmids can carry genes for co-resistance to other antibiotics such as aminoglycoside, fluoroquinolones, tetracyclines, chloramphenicol and sulfamethoxazole-trimethoprim. Antibiotic selection for such isolates thus becomes a therapeutic challenge. The widespread clinical use of broad spectrum β-lactam antibiotics has led to a marked increase in the incidence of ESBL producing gram negative microbes [Ma *et al.*, 2009; Shakil *et al.*, 2010]. The majority of ESBLs are derived from the widespread broad spectrum β-lactamases TEM-1 and SHV-1 [Wright *et al.*, 2005, Gazouli *et al.*, 1998]. However, the prevalence of occurrence of these organisms varies significantly in different geographical regions. CTX-Ms are a class of ESBLs that are name after the antibiotic 'cefotaxime'. CTX-Ms have become the most prevalent ESBLs worldwide [Rodríguez-Baño *et al.*, 2010], but have most often been associated with focal outbreaks in Eastern Europe, South America, Southeast Asia-Japan and recently in India with many variants described [Rodriguez-Baño *et al.*, 2006; Akram *et al.*, 2011].CTX-M type ESBL are mainly found in *Escherichia coli* strains and CTX-M producers are extremely common in UTIs [Akram *et al.*, 2007]. The CTX-M-15 enzyme in particular is increasingly being reported among *E.coil* isolates from northern India. In view of this background we have initiated this study to characterize the bacterial pathogens in patients having gram negative septicemia. Further, to evaluate the antimicrobial resistance and underlying molecular mechanisms in these strains. The

present study was undertaken; to assess the prevalence of plasmid mediated ESBL spread in Medical ICU of a teaching hospital in North India.

3.2 Experimental outline

The present study was conducted in Interdisciplinary Biotechnology Unit of AMU, India in collaboration with the department of Medicine, of a tertiary care teaching Hospital Aligarh, India. Inclusion criteria: patients having clinical features and bacteriological evidence of sepsis, septic shock, multi-organ dysfunction and/or systemic manifestation of gram negative septicemia. Exclusion criteria: Immunocompromised, patients with disseminated TB, HIV and patients on steroids were excluded from the study. Informed written consent was given by all study subjects and the study was approved by Institutional Ethics committee. Study design: Prospective, observational, non-interventional study. Isolation and identification of microorganism: On admission appropriate culture: urine, sputum, pus, urinary catheter tip, tracheal aspirate was sent in vial using standard techniques. Standard methods for isolation and identification of bacteria were used throughout the study [Sutter *et al.*, 1985]. The identified bacteria were also confirmed by the selective media of the Hi-crome series (Hi-media, Mumbai, India). Antimicrobial susceptibility testing of the isolates was performed by the CLSI standard disc diffusion method as outlined in section 2.2.2. The ESBL phenotypic confirmatory test was performed for all the probable ESBL-producers as mentioned in section 2.2.2. A search for ***bla*** $_{CTX-M}$, ***bla*** $_{SHV}$ and ***bla*** $_{TEM}$ gene was made among *E.coli*, *Klebsiella* spp. and *Pseudomonas* using both plasmid and genomic DNA and typing was performed by ERIC-PCR, as outlined in section 2.2.7. Conjugation studies for marker transfers were also carried out on these strains by both matting method as described in section 2.2.4.

3.3 Results

Total 70 patients with mean age of 55.94 ± 10.77 years, predominantly men (70%) and women (30%) were studied. On clinical evaluation, urinary tract (61.4%) was the most common site of infection, followed by respiratory tract (28.6%) skin and soft tissue (10%). The most common organism encountered for sepsis was *E.coli* 41(58.6%) followed by *Klebsiella* spp. 23(32.9%) and *Pseudomonas* 6 (8.6%). In patients having urosepsis most common organism responsible was *E.coli* 35(81.4%) followed by

Klebsiella spp. 6(14%) and *Pseudomona* 2(4.6%). In patient having pneumonia with sepsis the most common organism encountered was *Klebsiella* spp. 15 (75%) followed by *Pseudomona* 4(20%) and *E.coli* 1(5%). In patients having diabetic foot with sepsis in the study group most common organism encountered was *E.coli* 5(71%) followed by *Klebsiella* spp. 2(28.6%).The most common co-morbidity was diabetes mellitus 35 (50%), followed by benign Prostatic Hypertrophy 10(14.3%) and Chronic Obstructive Airway Disease 3(4.3%) (Table 3.1).

In the study group, *E.coli, Klebsiella* spp and *Pseudomonas* found to be resistant to gatifloxacin in 31.7%, 17.4% and 66.75% patients respectively. *E.coli, Klebsiella* spp and *Pseudomonas* were found to be resistant to the amikacin in 43.9%, 69.6% and 16.7% patients respectively. *E.coli* showed 7.3% intermediate resistance to amikacin. All the three organisms were 100% resistant to ampicillin and co-trimoxazole. *E.coli* and *Pseudomonas* showed 0% resistance to imipenem on the other hand *Klebsiella* spp. Showed 4.3% resistance. *E.coli, Klebsiella* spp and *Pseudomonas* were found to be resistant to meropenem in 9.7% and 50% patients respectively. *E.coli* and *Klebsiella* spp showed intermediate resistance to meropenem in 4.8% and 47.8% patients respectively. Most of the gram negative organisms are sensitive to imipenem and meropenem. Almost all gram negative organisms (*E.coli, Klebsiella* spp and *Pseudomonas*) were resistant to co-trimoxazole. (Table 3.2)

In the present study 7.3% *E.coli* isolates showed ESBL production while 12.2% *E.coli* showed MBL production on the other hand *Klebsiella* spp and *Pseudomonas* showed no ESBL/MBL production. In the present study majority of the isolates encountered for gram negative sepsis was *E.coli* (58.6%) and 81.4% *E.coli* were isolated from patients having urosepsis.

In the present study only 3 (7.3%) *E. coli* strains had a phenotype consistent with production of an ESBL. These 3 *E. coli* isolates were screened for the presence of ceftrixone hydrolyzing β-lactamases (CTX-M) and bla_{TEM} and bla_{SHV} types by PCR using universal primers. Three isolates (UE-59, UE- 61, and UE -64) showed the presence of bla_{TEM}, and bla_{CTX-M} type enzyme, while all the three were found negative for bla_{SHV} (Figure 3.1 & 3.2). Furthermore, conjugation experiments using J-53 as recipient susceptible cells confirmed that these markers are present on plasmid.

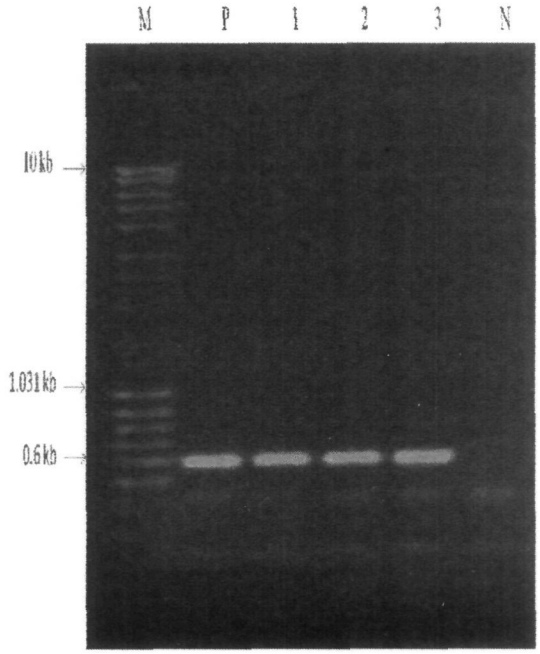


Figure 3.1. PCR amplification of *bla*$_{CTX-M}$ gene. Lane M: DNA ladder, Lane P: Positive control, Lane 1-3: *E. coli* clinical isolates (UE-59, UE-61, and UE-64) Lane N: Negative control.

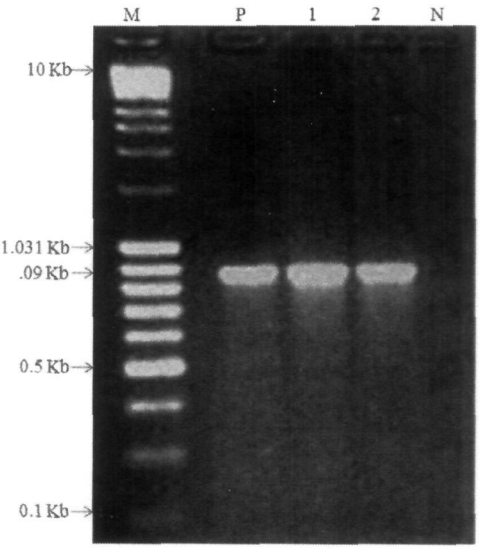


Figure 3.2. PCR amplification of *bla*$_{TEM}$ gene. Lane M: DNA ladder, Lane P: Positive control, Lane 1-3: *E. coli* clinical isolates (UE-59 and UE-64) Lane N: Negative control.

Table 3.1 Percent etiology and sources of sepsis

Source of Sepsis	*E.coli*	*Klebsiella Spp*	*Pseudomonas*	No=70 (100%)
Urosepsis	35(85.36)	6(26.08)	2(33.33)	43(61.4)
Pneumonia	1(2.49)	15(65.21)	4(66.66)	20(28.6)
Diabetic foot	5(12.19)	2(8.69)	0(0.00)	7(10)

Table 3.2 Percentage of resistance to the selected antimicrobial agents among the Gram negative bacteria

	β-Lactam		Aminoglycosides			Flouroquinolne			Cycline	Cloxacillin		Cephalosporin					Carbapene		
Pathogens (ESBL Producing)	A	P	G	Tb	Ak	Na	Cip	Gat	Te	Cot	Cox	Cz	Cn	Ce	Ci	Cpm	Mrp	Etp	Ipm
E.coli **(41 Isolates)**	100	100	43.9	51.0	43.9	100	100	31.7	95.1	100	100	100	100	100	100	100	9.7	0.0	0.0
Klebsiella **(23 Isolates)**	100	100	28.0	100	69.6	100	100	17.4	87.0	100	10.0	91.3	91.3	91.4	95.6	95.6	17.4	4.3	4.3
Pseudomonas **(6 Isolates)**	100	16.0	12.0	12.0	16.7	100	100	66.7	83.3	100	58.0	100	100	100	83.3	100	50.0	33.0	0.0

Ampicillin(A),Penicillin(P),Gentamicin(G),Tobramycin(Tb), Amikacin(Ak),Nalidixic-acid(Na),Ciprofloxacin(Cip),Gatifloxacin(Gat), Tetracycline(Te), Cotrimoxazole(Cot), Cloxacillin(Cox), Cefazolin(Cz), Cephoxitin(Cn), Cephotaxime(Ce), Ceftriaxone(Ci), Cefepime(Cpm), Meropenem(Mrp), Ertapenem(Etp), Imipenem(Ipm).

3.4 Discussion

Gram-negative sepsis is a significant problem in hospitalized and community- dwelling patients. Although the percentage of hospital-acquired bloodstream infections (BSIs) associated with gram-negative bacilli has decreased, these organisms now pose serious therapeutic problems because of multidrug resistance [Suarez *et al.*, 2005].

The treatment of gram-negative bacteremia is increasingly complicated by the occurrence of multidrug resistant gram negative bacilli strains. Over production of chromosomal β-lactamases by *Enterobacter* spp. has become one of the most common mechanisms of resistance to third generation cephalosporins [Goossens *et al.*, 2005]. In our study group most common organism encountered for sepsis was *E.coli* 41(58.6%) followed by *Klebsiella* spp. 23(32.9%) and *Pseudomonas* 6(8.6%). Similar results for the most common isolates were identified in community acquired sepsis in an Italian study: *E.coli* (76%); *P. aeruginosa* (7.9%); *K. pneumoniae* (5.4%); *Proteus mirabilis* 4.2%; *Enterobacteriace* species 3.7 percent [Luzzaro *et al.*, 2002]. UTI remains the commonest type of community acquired gram negative sepsis, especially in developing countries. A similar study has been performed earlier where Taylor *et al* showed most common organisms responsible for nosocomial urosepsis were *E.coli* (36.8%) and *Pseudomonas* 8.9% [Taylor *et al.*, 1996]. In our study most common organism isolated from patients having pneumonia was *Klebsiella* spp. 15(75%) followed by *Pseudomonas* 4(20%) and *E.coli* 1(5%). A similar study done by Feldeman *et al* who found 31.9% isolates were *Klebsiella* spp and 8.9% were *Pseudomonas* [Feldman *et al.*, 1995]. In the present study *E.coli, Klebsiella* spp and *Pseudomonas* were found to be 100%, 95% and 100% respectively resistant to cefazolin and cefuroxime. For ceftazidime, *Ecoli* was found to be 100% resistant, *Klebsiella* spp. Showed 91.4% resistance and *Pseudomonas* showed 83.3% resistance. Our findings are comparable to study conducted by Kumari *et al* [Kumari *et al.*, 2007]. In the present study 7.3% *E.coli* isolates showed ESBL production on the other hand *Klebsiella* spp and *Pseudomonas* showed no ESBL/MBL production. While MBL production was seen in 12.2% *E.coli.* Extended spectrum β-lactamase (ESBL) and metallo β-lactamase (MBL) production is a matter of great concern in the field of microbial drug resistance. ESBL represents a major threat among resistant

bacterial isolates of *Enterobacteriaceae* [Paterson *et al.*, 2005].This renders powerful antibiotics, like expanded spectrum cephalosporins, carbapenems and monobactams ineffective. India has significantly high pool of ESBL infection, reflecting poor standards of hygiene and inappropriate use of antibiotics, as compared to other developed societies. The source of ESBL producing *Enterobacteriaceae* differ, while in other countries it is mainly hospital acquired, in India it is mostly community acquired [Canton *et al.*, 2008]. Different groups of ESBLs, have been described and classified according to their amino acid sequences, like SHV-1, TEM-2, TEM-3 etc. Until the end of the 1990s, most of the ESBLs detected were SHV and TEM types, mostly associated with nosocomial outbreaks [Livermore *et al.*, 2005]. In 1989 simultaneous detection of ESBLs producers have been identified in different country of Europe. CTX-M was named because most of the enzymes within this family confer resistance predominantly to cefotaxime rather than ceftazidime [Eckert *et al.*, 2006]. The CTX-M ESBLs have since been detected in many species of *Enterobacteriaceae*, currently the CTX-M family includes more than 100 vairents which may be grouped on the basis of sequence similarity in to 4 distinct subtypes epitomized by CTX-M-1, CTX-M-2, CTX-M-8, and CTX-M-9. The $bla_{\text{CTX-M-15}}$ gene borne on plasmid in *Enterobacteriaceae,* was first detected in India, which is now the globally dominant ESBL [Walsh *et al.*, 2007]. Unlike in other countries e.g. Spain, France, where members of multiple CTX-M lineages have been reported, a virtually absolute prevalence of the members of the CTX-M-1 lineage was observed in this study [Cantón *et al.*, 2006]. Recent reports have shown a rapid and alarming dissemination of *Enterobacteriaceae* producing ESBLs of the CTX-M type in certain counties including India and have become the most prevalent ESBL type worldwide [Walsh *et al.*, 2007]. The majority of these isolates are now recovered from community patients, most of them with urinary tract infection [Eshwarappa *et al.*, 2011]. Unlike that of TEM or SHV type ESBLs, the population structure of CTX-M producing isolates is complex and is associated with the spread of specific plasmid and /or other mobile genetic elements rather than clonal epidemics [Mendonca *et al.*, 2007].

Bacterial resistance to commonly used antibiotics, in a resource crunched developing country like India, threatens the basic health delivery systems, affecting millions. There are alarming reports about serious consequences of antibiotic resistance. However, there

is still a scarcity of data on the magnitude of antibiotic resistance, especially in Asia-Pacific region.

3.5 Conclusion

This work has provided insights into molecular epidemiology of escalating antimicrobial resistance, especially prevalent in ESBL producing *Enterobacteraceae* in India. Continueing surveillance will be necessary to monitor the evolution of ESBL and to authenticate whether the CTX-M type ESBLs will eventually prevail over the bla_{TEM}-type ESBLs, which are still widespread, especially in some areas. Our findings uphold the increasing role of the bla_{CTX-M} β-lactamases in antibiotic resistance and stress upon the significance of appropriate empirical treatment for infections caused by coliforms, especially during septicemia. This study confirms that horizontal gene transfer of antimicrobial resistance markers is the most prevalent among *Enterobacteraceae* in the community setting. Further studies are needed, however, to determine whether antibiotic policies or other measures can halt or lower the level of horizontal gene transfer that occurs in a hospital or community setting.

Chapter-4

Epidemiology and risk factors associated with CTX-M-3, TEM-1 and SHV-1 producing K. pneumoniae strains isolated from neonatal intensive care unit of North Indian hospital

4.1 Introduction

Extended spectrum β-lactamase (ESBL) producing *Klebsiella pneumoniae* constitute one of the most common Gram negative bacteria showing multiple antibiotic resistances worldwide [Livermore *et al.* 2007]. However, there is an epidemiological change in the community over the last 20 years with the emergence of virulence. An increasing prevalence of extended spectrum β-lactamase producing *Klebsiella pneumoniae* (EPK) was reported during the past decade as reported by Centers for Disease Control and Prevention National Nosocomial Infection Surveillance study, particularly in intensive care unit (ICUs) [Fridkin *et al.*, 1999]. ESBLs (***bla***$_{CTX-M}$, ***bla***$_{TEM}$ and ***bla***$_{SHV}$) producing *K. pneumoniae* strains are spread throughout hospital settings, and become predominant in the community [Jacoby *et al.*, 1997; Cabrera *et al.*, 2009]. The worldwide spread of resistant gene marker is facilitated by horizontal gene transfer mechanisms. Antibiotic selection for such infective strains thus becomes a therapeutic challenge. Outbreaks by ESBL producing organisms have been described frequently in NICU settings [Mirelis *et al.*, 2003]. Clinical conditions and treatments predisposing to infection by such pathogens have been investigated but very few studies were able to identify independent risk factors and there is a paucity of ESBLs data concerning neonates, particularly in India. In view of the present situation, we initiated our study to understand the mode of mechanism among the *K. pneumoniae* isolates, circulating in NICU, Aligarh hospital of north India and to identify the various risk factors associated with acquisition of these infections caused by ESBL producers.

4.2 Experimental outline

Analysis of different risk factors like socioeconomic status, educational status and epidemiological like gender, mode of delivery, age, gestational age, birth weight (patients prior to infection, and severity of illness), maternal anemia, maternal intrapartum symptomatic infections (was define as receipt of at least 1 dose of antibiotic therapy during labor and considered an opportunity to provide antibiotics when mother was in the hospital after the risk condition and at least 1 hour before delivery), leaking per-vagina,

bleeding per-vagina, prolonged rupture of membrane unclear vaginal examination of mother, infection carriage (throat, nasal, eyelid, and urinary catheter at the time of infection), length of hospital stay before the onset sepsis in NICU and included instruments (mechanical ventilator, radiant wormer, phototherapy, cot, stethoscope, refrigerator and weighing machine). Patients selected for analysis were those admitted in the active surveillance system (NICU stay >48 h and weekly surveillance swabs taken at least once). Neonates admitted to the ward before January 2006 and discharged after February 2009 was excluded. A total of 18 risk factors were analyzed. Descriptive analyses were used for socioeconomic status, educational status and epidemiological data in terms of percentage between two groups of patients. Student t-test and Fishers test for univariate analysis were performed to explore the potential risk factors for ESBL and non ESBL producer strains. Odds ratio (OR) and 95% confidence intervals CI (95%) were also performed as mentioned in section 2.2.3. Normally distributed continuous variable was analyzed using student t-test were appropriate. The resistance rate was calculated as the number of resistant strains divided by the total number of strains. The difference was considered statistically significant if p-values were ≤ 0.05. All p-values were two tailed and statistical analysis was performed by Statistical Package for the Social Sciences (SPSS Inc. Chicago, USA) version 16.0.

Bacterial identifications were confirmed as mentioned in the previous study (Chapter 3). Antimicrobial susceptibility testing of the isolates was performed by the CLSI standard disc diffusion method as outlined in section 2.2.2. MICs of cephalothin, cefazolin, cefuroxime, cephoxitine, ceftriaxone, ceftazidime, cefotaxime and cefepime were determined by the CLSI microbroth dilution method. The ESBL phenotypic confirmatory test was performed for all the probable ESBL producers as mentioned in section 2.2.2. A search for $\boldsymbol{bla}_{\text{CTX-M}}$, $\boldsymbol{bla}_{\text{TEM}}$ and $\boldsymbol{bla}_{\text{SHV}}$ genes and integrons was carried out using the primers consisted of following oligonucleotide specific primer in table 2.1 made among these strains using both genomic and plasmid DNA and typing was performed by ERIC-PCR, as outlined in section 2.2.7. PCR-based replicon typing was carried out on 11 non-conjugative and conjugative plasmids. The 18 sets of primer (Table 2.1) were used to target HI1, HI2, I1-Iγ, X L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, FIIAs, F, K and B/O replicons (incompatibility) were used in separate PCR reactions described elsewhere

[Elhani *et al.*, 2010]. MLST was done according to the protocol described in section 2.2.12 and sequence typing was analyzed according to the site (http://www.pasteur.fr/recherche/genopole/PF8/mlst/kpneumoniae.html) on 4 isolates of *K. pneumoniae* selected on the basis of ERIC and replicon typing. Sequencing was done as mentioned in section 2.2.10. Transmissibility of bla_{CTX-M} markers was checked by conjugation as described in section 2.2.4.

4.3 Results

A total of 3500 neonates that were screened for the infection in NICU over the period of 3 yerars, 970 (27.71%) were found to have clinically suspected culture, while 365 (37.62 %) of them were positive for neonatal infection. Among 365 clinical isolate, 103 (28.21 %) were EPK while 262 (71.78 %) were non EPK.

Of 103 EPK strains, 64 (62.13%) isolates of *K. pneumoniae* were found in low socioeconomic status group. Whereas, 25 (24.27%) and 14 (13.95%) were observed among middle and upper income group respectively (Table 4.1). Among 103 isolates of ESBL carriers, 37 (35.92%) were illiterate, 47 (45.63%) were literate and 19 (18.44%) were educated. On the basis of sex ratio, among these ESBL producers, 79 (76.69%) were male and 24 (23.20%) of them were found among females. Furthermore, the study also revealed that, 72 (69.69%) case were found to be cesarean while remaining were normal births. A total of 76 (65.04%), age wise ESBL carriage were observed among the neonates of 0-5 days whereas, 36 (34.95%) were present in the neonates of 6-30 days. Our study further revealed that on the basis of gestational period, 81 (78.64%) ESBL producers were observed as preterm babies and 22 (21.35%) as term babies. Classification based on onset sepsis reveled 80 (77.66%) isolates as early onset sepsis and 23 (22.33%) isolates as late onset sepsis. The present study also showed that according to the birth weight, 62 (60.19%) clinical isolates were obtained from neonates weighing less than 1500g whereas, 41 (39.80%) neonates were of more than 1500g. Our data showed that occurrence of EPK in maternal anemia, maternal intrapatum symptomatic infection, leaking per vagina, bleeding per vagina, prolonged rupture of membrane, and unclear vaginal examination of mother were 38 (36.89%), 11 (10.67%), 11 (10.67%), 12

(11.65%), 21 (20.38%), 43 (41.74%), and 70 (67.96%) respectively (Table 4.1). During the study period, 25 (24.27%), 37 (35.92%), 7 (6.70%) and 20 (19.21%) EPK were responsible for infection carriage in throat, nasal, conjunctivitis and urinary tract infection (UTI) respectively. Furthermore, neonates staying for 1-5 days in hospital, showed 66.99% of infection while neonates staying for 6-30 days in the hospital showed 33.00% infection (Table 4.1). Of 103 EPK isolates from NICU, 30 (29.12%) isolates were of environmental origin. These 30 isolates were distributed among, ventilator, radiant wormer, phototherapy, cot, stethoscope, refrigerator and weighing machine with distribution figures, 2 (1.94%), 4 (3.88%), 7 (6.79%), 9 (8.73%), 5 (4.85%), 1 (0.97%) and 2 (1.94%) respectively (Table 4.1).

A total of 365 isolates of *K. pneumoniae* were recovered during the study period. Comparative susceptibility test among EPK and non EPK with antibiotics (Table 4.2) revealed that ESBL positive isolates of *K. pneumoniae* (EPK) had significantly higher resistance against these antibiotics. In EPK, our study demonstrated that, higher and lowest percent of resistant strains were observed against penicillin (86.40%) and pipracillin (57.28%) respectively among the β-lactum groups (Table 4.2). Moreover, 79.61% and 32.03% resistance was observed against tobramycin and amikacin respectively. Our study further revealed that among the fluoroquinolone, highest and lowest percent resistance was observed against nalidixic acid (84.46%) and gatifloxacin (27.18%), respectively. Furthermore, susceptibility testing of these isolates revealed highest resistance against cotrimoxazol and tetracycline. A total of 43 (41.75%) EPK strains were found resistant against cefepime (Table 4.2). Some of the EPK isolates from neonates of various risk status, shared resistance against meropenem.

EPK strains isolated from NICU, showed presence of multiple plasmids of different molecular size (Table 4.3). Among 103 EPK isolates with two or three plasmids, the presence of small size transferable plasmids (28 kb) was detected in 21 (20.38%) EPK. 74 (71.84%) of these EPK strains harbored a conjugative mega plasmid. These mega plasmids bear gene imparting resistance against extended-spectrum cephalosporins. The transconjugants were also detected for their susceptibility against various antibiotics of β-lactam, aminoglycoside and fluroquinolone group using disk diffusion test and

microdilution method. The tranconjugants showed multidrug resistance (MDR) phenotype including resistance to all generation of cephalosporine.

ERIC-PCR was performed for the analysis of molecular characterization of *K. pneumoniae* strains which categorized 103 strains in 11 different phylogenic groups (Figure 4.1). Also, the 11 stains from each phylogenic group were further confirmed by ERIC-PCR as shown in figure 4.2. The A5 group was most frequently observed among the isolates (26 of 103 strains, 25.24%). The types of ESBL producing *K. pneumoniae are* summarized in table 4.1. Of 103 *K. pneumoniae* cases 19 had $bla_{CTX-M-3}$, 28 had bla_{TEM-1} and 17 were found to have bla_{SHV-1} type ESBLs. Integrons were also detected in all 11 strains with a consistent size of 530bp (Figure 4.3), representing each phylogenic group and was found as class I integron carrying *Int*-1 (Figure 4.4) and *Sul*-1 (Figure 4.5) genes of the expected sizes 845bp and 840bp, respectively. Amplified products for each bla_{CTX-M} (Figure 4.6), bla_{TEM} (Figure 4.7), and bla_{SHV} (Figure 4.8) were sequenced and analyzed using NCBI data base site (http/www.ncbi.nlm.nih.gov/).The plasmids carrying the $bla_{CTX-M-3}$ bla_{TEM-1} and bla_{SHV-1} genes were assigned to Inc/H12 (Figure 4.9), Inc/X (Figure 4.10) Inc/FIC (Figure 4.11), and Inc/FIB (Figure 4.12) replicon types. MLST analysis of 4 isolates revealed 3 different sequence types (STs), whereas, one of them was found untypeable (Table 4). Moreover, this analysis also showed dissemination of $bla_{CTX-M-3}$, bla_{TEM-1} and bla_{SHV-1} gene through *K. pneumoniae* clinical isolates of ST-64, ST-280 and environmental isolate of ST-51 in the NICU (Table 4.4 and figure 4.13-4.19).

In the univariate analysis of EPK, several study variables like maternal intrapartum symptomatic infection, prolonged rupture of membrane, length of stay in NICU and Carbapenem use (P=0.00) were found to be very closely associated with EPK and non-EPK acquisition status of neonates (Table 4.1).

Independent risk factors for EPK, included seven different types of variables associated with ESBL carriage (Table 4.1) under the socioeconomic status of lower income group (p=0.01), illiterate under the educational status (p=0.03), preterm under the gestational age (p=0.005), early onset sepsis under the onset sepsis (p=0.02), <1500g under the birth weight (p=0.04), nasal (p=0.03), conjunctivitis (p=0.02), UTI (p=0.04) under the infection carriage and instrumentation (p=0.03), ampicillin (p=0.02) and penicillin (p=0.01) under the β-lactam, amikacine under the aminoglycosides (p=0.04),

ciprofloxacin under the fluoroquinolone (p=0.04) as well as gatifloxacine under the fluoroquinolone (p=0.03) and cefepime under the cephalosporin 4th generation (p=0.02) respectively. We observed that use of 3rd and 4th generation of cephalosporine and carbapenems were significantly associated with infection of ESBL-producing organism in the univariate analysis, but no significance was observed in the multivariate analysis (Table 4.2).

Table 4.1 Characteristics of NICU patients and analysis of their risk factors associated with ESBLs producing *Klebsiella pneumoniae* strains

Study variables	ESBL producing *Klebsiella pneumoniae* N=103 & %(28.21)	Non ESBL producing *Klebsiella pneumoniae* N=262 & %(71.78)	p-value	OR (95% CI)	ERIC types of ESBL producers	Type of ESBLs
Socioeconomic status						
Lower income group	64(62.13)	128(48.85)	0.01	1.71(1.07-2.73)	A1,A2,A3,A4,A5,A9,A10,A11	*bla*CTXM-3, *bla*TEM-1, *bla*SHV-1
Middle income group	25(24.27)	80(30.53)	0.10	0.72(0.43-0.22)	A4,A5,A9,A10	*bla*CTXM-3, *bla*TEM-1
Upper income group	14(13.95)	54(20.61	0.09	0.60(0.31-1.14)	A3,A5,A6,	*bla*TEM-1
Educational status						
Illiterate	37(35.92)	125(47.70)	0.03	0.61(0.38-0.98)	A1,A3,A4,A5,A6,A10	*bla*CTXM-3, *bla*TEM-1, *bla*SHV-1
Literate	47(45.63)	98(37.47)	0.12	1.40(0.88-2.22)	A1,A4,A5,A6,A10,A11,	*bla*CTXM-3, *bla*TEM-1, *bla*SHV-1
Educate	19(18.44)	39(14.88)	0.34	1.29(0.07-2.36)	A4,A5,A11	*bla*TEM-1
Sex						
Male	79(76.69)	179(68.32)		1.00	A1,A2,A4,A5,A6,A10,A11	*bla*CTXM-3, *bla*TEM-1, *bla*SHV-1
Female	24(23.20)	83(31.67)	0.09	1.53(0.90-2.58)	A2,A3,A5,A9,A11	*bla*CTXM-3, *bla*TEM-1
Mode of delivery						
Cesarean	72(69.90)	157(59.92)		1.00	A1,A2,A3,A4,A5,A6,A9,A10	*bla*CTXM-3, *bla*TEM-1, *bla*SHV-1
Normal	31(30.09)	105(40.07)	0.07	1.55(0.95-2.53)	A4,A9,A11	*bla*CTXM-3, *bla*SHV-1
Age						
0-5 days	67(65.04)	163(62.21)		1.00	A1,A2,A4,A5,A6,A10	*bla*CTXM-3, *bla*TEM-1, *bla*SHV-1
6->30 days	36(34.95)	99(37.78)	0.55	1.13(0.70-1.82)	A1,A3,A11	*bla*CTXM-3
Gestational age						
Preterm	81(78.64)	169(64.50)		1.00	A1,A3,A4,A5,A6,A8	*bla*CTXM-3, *bla*TEM-1, *bla*SHV-1
Term	22(21.35)	93(35.49)	0.005	2.03(1.19-3.46)	A5,A8,A11	*bla*TEM-1
Onset sepsis						
Early	80(77.66)	173(66.03)		1.00	A1,A3,A4,A5,A9	*bla*CTXM-3, *bla*TEM-1, *bla*SHV-1
Late	23(22.33)	89(33.96)	0.02	1.78(1.05-3.03)	A5,A8	*bla*TEM-1
Birth weight						
<1500g	62(60.19)	186(70.99)		1.00	A1,A2,A4,A6,A9,A10	*bla*CTXM-3, *bla*TEM-1, *bla*SHV-1
>1500g	41(39.80)	76(29.00)	0.04	0.61(0.38-0.99)	A4,A5	*bla*TEM-1
Maternal anemia	38(36.89)	86(32.82)	0.39	1.19(0.74-1.92)	A1,A6	*bla*CTXM-3
Maternal intrapartum fever	11(10.67)	27(10.30)	0.85	1.04(0.49-2.18)	A7	*bla*SHV-1
Maternal intrapartum symptomatic infections	11(10.67)	05(01.90)	0.00	6.14(2.08-18.2)	A7	*bla*SHV-1
Leaking Per Vagina	12(11.65)	47(17.93)	0.11	0.60(0.31-1.19)	A4	*bla*SHV-1
Bleeding Per Vagina	21(20.38)	67(25.57)	0.23	0.74(0.43-1.29)	A8,A11	*bla*SHV-1

Contd----

Prolonged rupture of membrane	43(41.74)	89(33.96)	0.00	3.98(2.23-7.12)	A1,A2,A5,A9,A11	*bla*CTXM-3, *bla*TEM-1
Unclear vaginal examination of mother	70(67.96)	47(17.93)	0.76	0.90(0.49-1.66)	A1,A3,A4,A6,A10	*bla*CTXM-3, *bla*SHV-1
Infection carriage						
Throat	25(24.27)	82(31.29)	0.15	0.70(0.41-1.18)	A1,A11	*bla*CTXM-3
Nasal	37(35.92)	67(25.57)	0.03	1.63(1.00-2.66)	A1,A2,A5,A10	*bla*CTXM-3, *bla*TEM-1
Conjunctivitis	07(06.70)	39(14.88)	0.02	0.41(0.18-0.96)	A5	*bla*TEM-1
UTI	20(19.41)	31(11.83)	0.04	1.79(0.97-3.32)	A5,A11	*bla*TEM-1
Length of stay in NICU						
1-5 days	34(33.00)	103(39.31)		1.00	A1,A2,A3,A4,A5	*bla*CTXM-3, *bla*TEM-1, *bla*SHV-1
6->30 days	69(66.99)	159(60.68)	0.00	3.13(1.94-5.06)	A8,A10,A11	*bla*SHV-1
Instrument/Equipment						
Ventilator	02(01.94)	03(01.14)			A3	*bla*TEM-1
Radiant wormer	04(03.88)	02(00.76)			A1	*bla*TEM-1
Photo therapy	07(06.79)	09(03.43)			A2,A11	*bla*TEM-1
Cot	09(08.73)	06(02.29)			A1,A11	*bla*TEM-1
Stethoscope	05(04.85)	09(03.43)			A1	*bla*TEM-1
Refrigerator	01(00.97)	02(00.76)			A2,A11	*bla*TEM-1
Weighing machine	02(01.94)	01(00.38)	0.03	0.31(0.26-0.35)	A1	*bla*TEM-1

Table 4.2 Resistance pattern of ESBL and non ESBL producing *Klesiella pneumoniae* strains from NICU of Aligarh hospital

Antibiotic groups	Antibiotics		% Resistance to antibiotics of ESBL N=103 (28.21%)	% Resistance to antibiotics of Non ESBL N=262 (71.78%)	p-value	OR (95% CI)	MIC range of ESBL producer	MIC range of Non ESBL producer
Aminoglycosides		G	59(57.28)	130(49.61)	0.16	1.36(0.86-2.15)	- -	- -
		Tb	82(79.61)	196(74.80)	0.41	1.26(0.72-2.20)	- -	- -
		Ak	33(32.03)	113(43.12)	0.04	0.62(0.30-1.00)	- -	- -
Fluoroquinolones		Na	87(84.46)	211(80.53)	0.36	1.31(0.71-2.43)	- -	- -
		Cf	73(70.83)	157(59.92)	0.04	1.62(0.99-2.66)	- -	- -
		Gf	28(27.18)	101(38.54)	0.03	0.59(0.36-0.98)	- -	- -
β-Lactams		A	99(96.11)	232(88.54)	0.02	3.20(1.09-9.32)	- -	- -
		P	96(93.20)	217(82.82)	0.01	2.84(1.23-6.53)	- -	- -
		Pc	83(80.58)	198(75.57)	0.33	1.34(0.46-2.35)	- -	- -
Others		T	81(78.64)	190(72.51)	0.23	1.39(0.81-2.40)	- -	- -
		Co	94(91.26)	223(85.11)	0.08	1.82(0.85-3.92)	- -	- -
Cephalosporins	$1^{st}g$	Ch	89(86.40)	217(82.82)	0.43	1.31(0.68-2.50)	62.50-250	31.25-125
		Cz	84(81.55)	205(78.24)	0.56	1.22(0.68-2.10)	31.25-125	15.62-125
	$2^{n}g$	Cu	80(77.66)	199(75.95)	0.68	1.15(0.67-1.98)	62.50-250	62.50-250
		Cn	73(70.87)	199(75.95)	0.35	0.77(0.46-1.28)	31.25-125	15.62-125
	$3^{rd}g$	Ca	71(68.93)	189(72.13)	0.07	0.62(0.37-1.04)	62.50-250	31.25-125
		Ce	70(67.98)	178(67.93)	0.80	0.91(0.56-1.48)	62.50-250	31.25-125
		Ci	70(67.98)	171(65.26)	0.71	1.12(0.69-1.83)	15.625-250	07.81-31.25
	$4^{th}g$	Cpm	43(41.74)	78(29.77)	0.02	1.69(1.05-2.71)	07.81-125	07.81-31.25
Carbapenems		Imp	01(00.97)	00(00.00)	0.00	3.56(1.10-1.43)	02-15.625	02-15.625
		Ert	02(01.94)	00(00.00)	0.00	3.59(1.11-1.44)	02-15.625	02-15.625
		Mr	39(37.86)	22(08.39)	0.00	6.64(3.68-12.0)	02-62.50	02-62.50

Generation (g), Gentamicin (G), Tobramycin (Tb), Amikacin (Ak), Nalidixicacid (Na), Ciprofloxacin (Cf), Gatifloxacin (Gf), Ampicillin (A), Penicillin (P), Pipracillin (Pc), Tetracycline (T), Co-trimoxazole (Co), Cephalothin (Ch), Cefazolin (Cz), Cefuroxime (Cu), Cephoxitine (Cn), Ceftazidime (Ca), Cephotaxime (Ce), Ceftriaxone (Ci), Cefepime (Cpm), Imipenem (Imp), Ertapenem (Ert), Meropenem (Mr).

Table 4.3 Characteristic of selected EPK isolates representing incompability, multilocus sequence typing analysis marker transfer in transconjugants

Starin No	113	229	277	295
Specimen Sample	UTI	Radiant wormer	Conjunctivitis	Refrigerator
ERIC Types	A1	A3	A5	A6
Number of Plasmids and size	154kb,64kb,28kb	154kb,64kb	154kb,64kb	154kb,64kb
Resistant determinant ESBL Types	$bla_{CTX-M-3}$,bla_{TEM-1},bla_{SHV-1}	$bla_{CTX-M-3}$,bla_{TEM-1}	$bla_{CTX-M-3}$,bla_{TEM-1},bla_{SHV-1}	$bla_{CTX-M-3}$,bla_{TEM-1},bla_{SHV-1}
Plasmids size in transconjugants	154kb,64kb,28kb	154kb,64kb	154kb,64kb	154kb,64kb
Marker transfer	$bla_{CTX-M-3}$,bla_{TEM-1},bla_{SHV-1}	$bla_{CTX-M-3}$,bla_{TEM-1}	$bla_{CTX-M-3}$,bla_{TEM-1}	$bla_{CTX-M-3}$,bla_{TEM-1}
Plasmid incompatibility groups	Inc/FIC	Inc/H12	Inc/X	Inc/FIB
MLST alleles	2-1-19-1-10-1-37	2-1-13-10-17-1-19	2-1-2-1-10-4-46	----------
ST	64	51	280	----------

LST, multilocus sequence typing; ST, sequence typing; UTI, urinary tract infection.

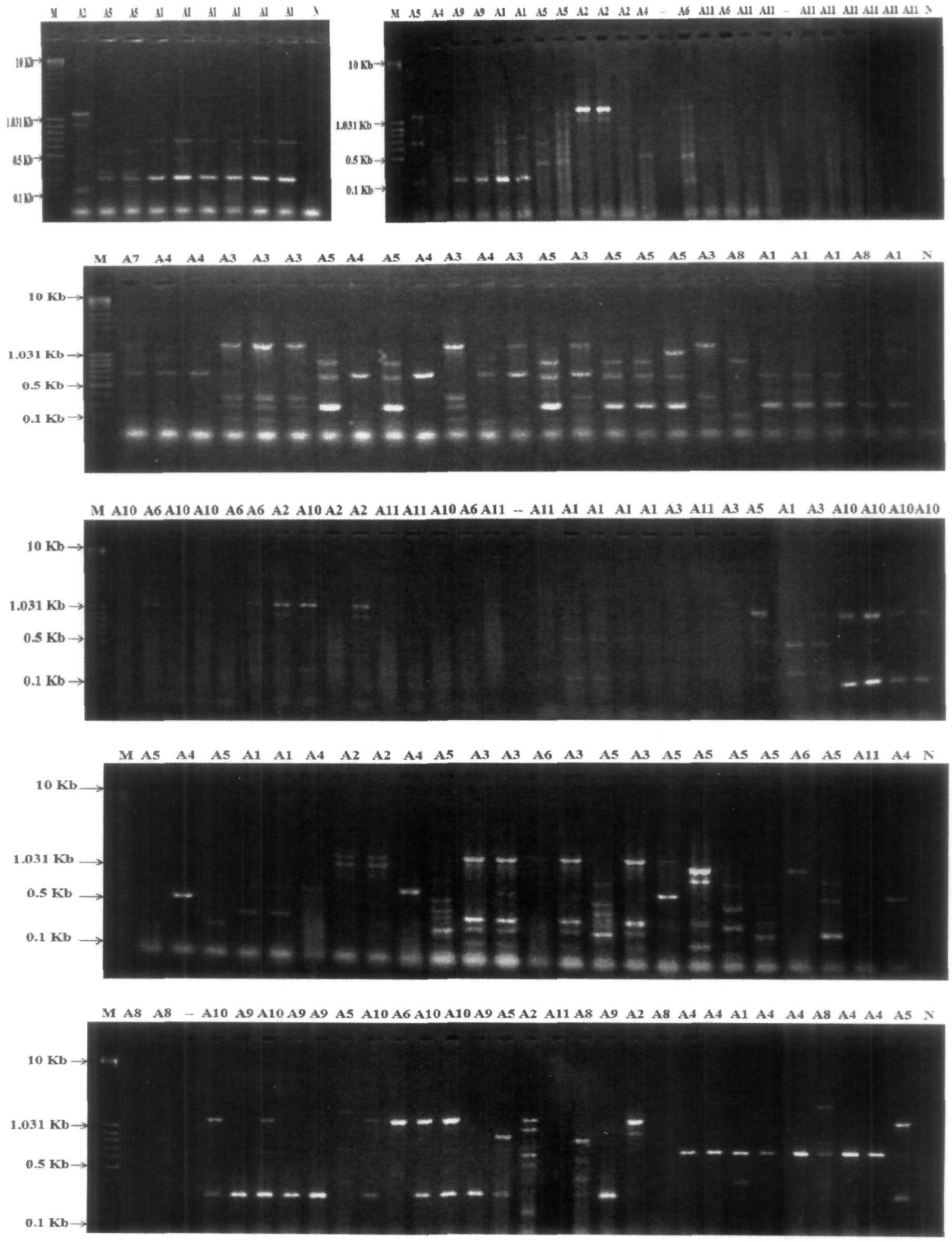


Figure 4.1 ERIC profiles of ESBL producing *K. pneumoniae* isolates from NICU. M stands for marker (the first lane of each figure), A1-A11 represents different ERIC typed clinical isolates and N stands for negative control (the last lane of each figure)

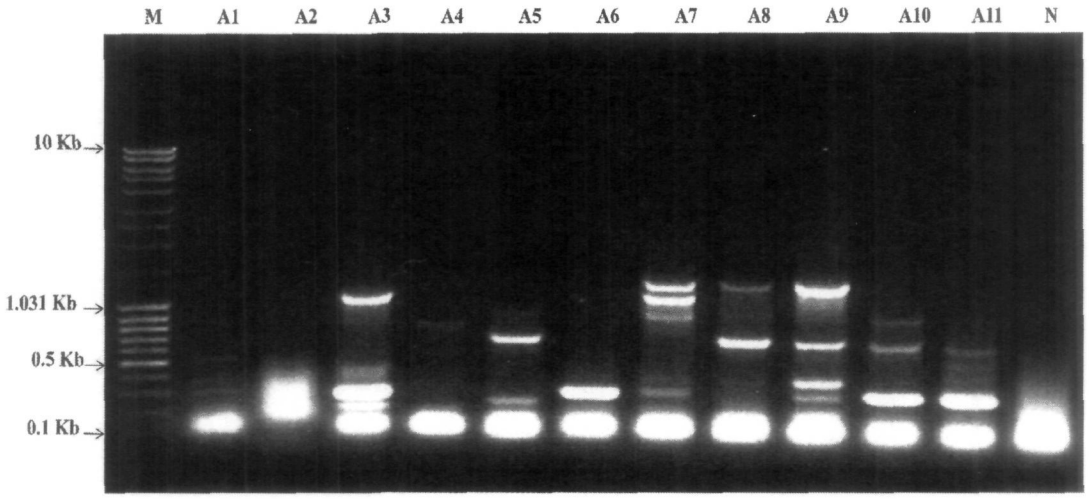


Figure 4.2 ERIC profile for reconfirmation of 11 different phylogenic groups of ESBL producing *K. penumoniae* in NICU. M stands for marker (the first lane of each figure), A1-A11 represents different ERIC typed clinical isolates and N stands for negative control (the last lane of each figure)

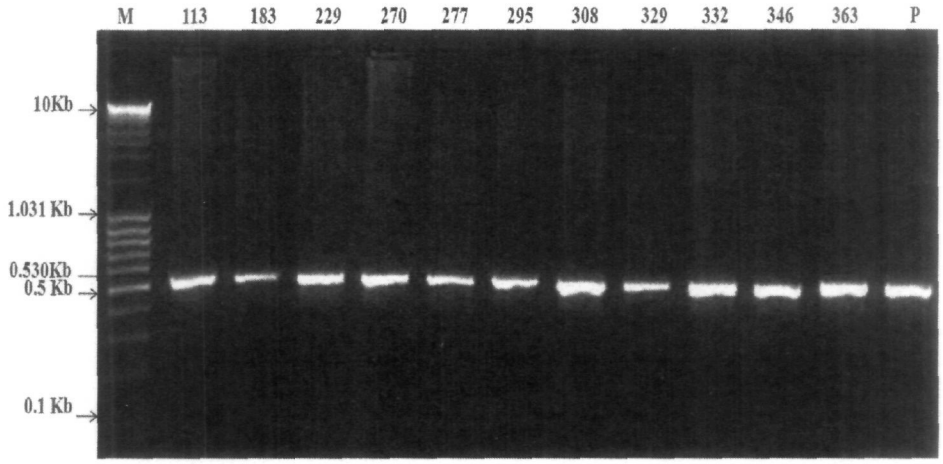


Figure 4.3 PCR amplification of 5'-CS-3'CS conserved sequences of integron. Lane M: 10 Kb DNA ladder, lane 1-11 *K. penumoniae* clinical isolates (113, 183, 229, 270, 277, 295, 308, 329, 332, 346, 363) and Lane P: positive control.

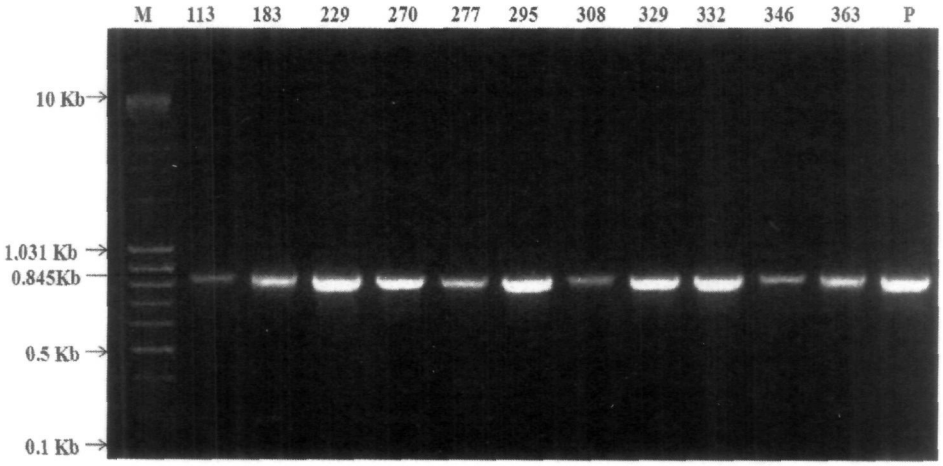


Figure 4.4 Integron mapping by PCR amplification of Int-1 gene. Lane M: 10 Kb DNA ladder, 1-11 *K. penumoniae* clinical isolates (113, 183, 229, 270, 277, 295, 308, 329, 332, 346, 363) and Lane P: positive control.

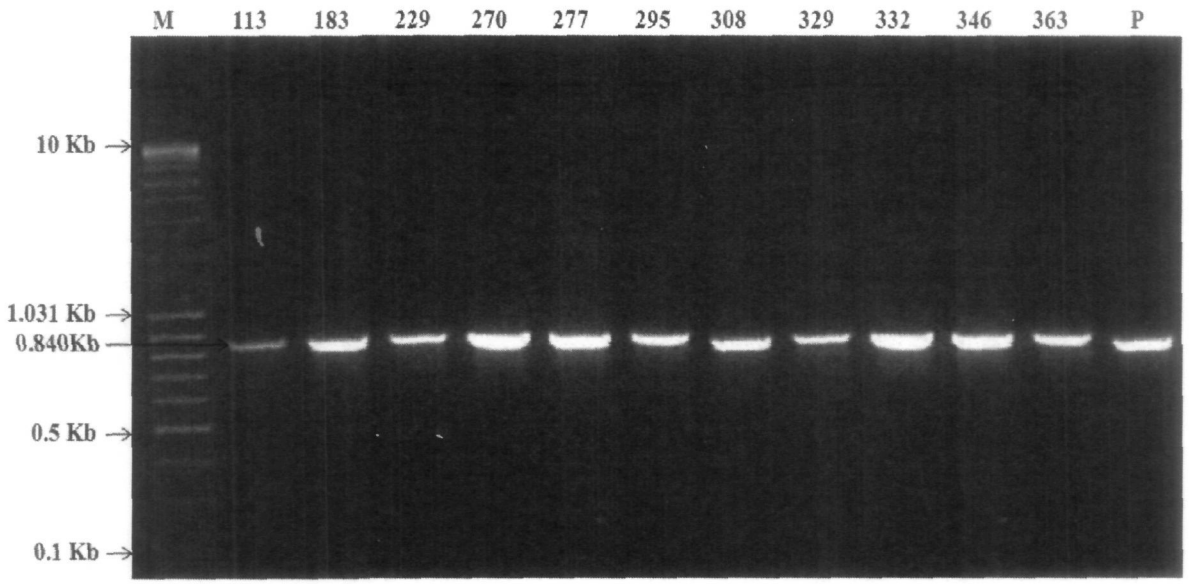


Figure 4.5 Integron mapping by PCR amplification of Sul-1 gene. Lane M: 10 Kb DNA ladder, 1-11 *K. penumoniae* clinical isolates (113, 183, 229, 270, 277, 295, 308, 329, 332,346, 363) and Lane P: positive control.

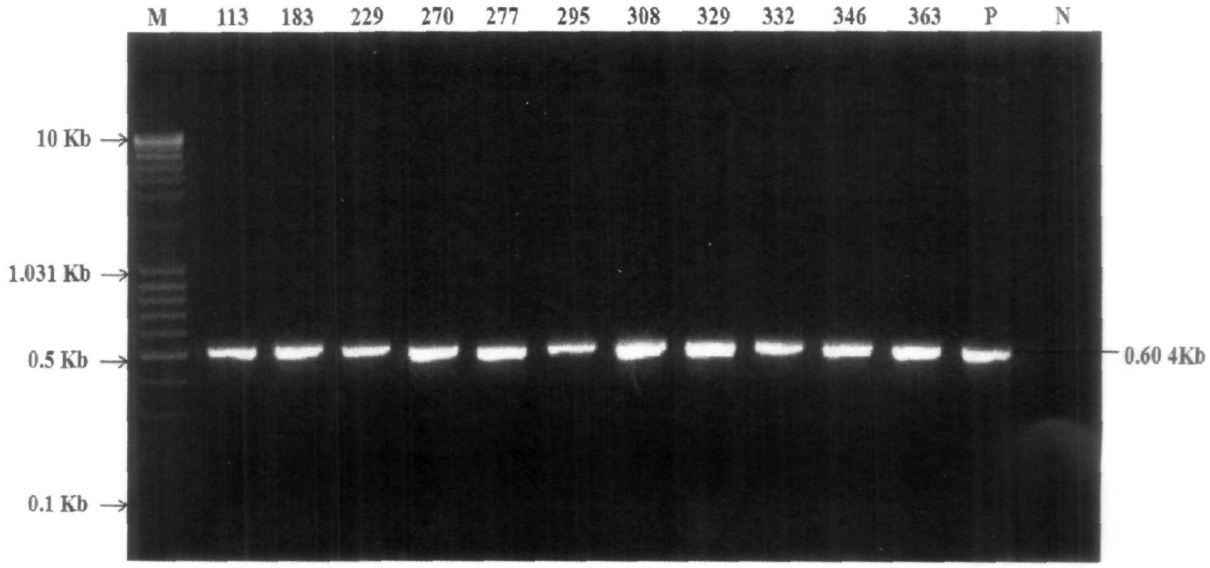


Figure 4.6 PCR amplification of CTX-M gene, Lane M: 10 Kb DNA ladder, 1-11 *K. penumoniae* clinical isolates (113, 183, 229, 270, 277, 295, 308, 329, 332, 346, 363), Lane P: positive control Lane N: negative control.

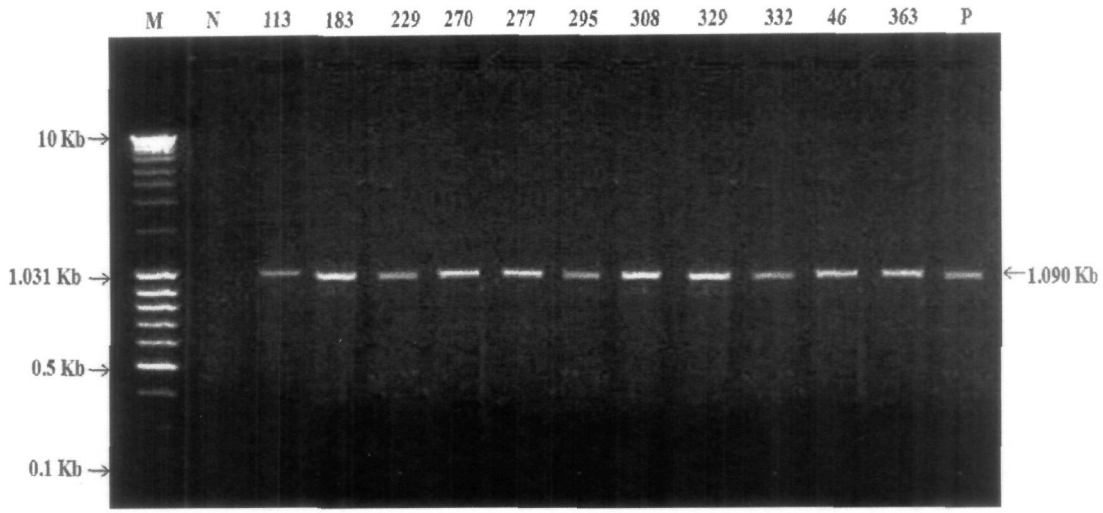


Figure 4.7 PCR amplification of TEM gene, Lane M: 10 Kb DNA ladder, Lane N: negative control 1-11 *K. penumoniae* clinical isolates (113, 183, 229, 270, 277, 295, 308, 329, 332, 346, 363), Lane P: positive control

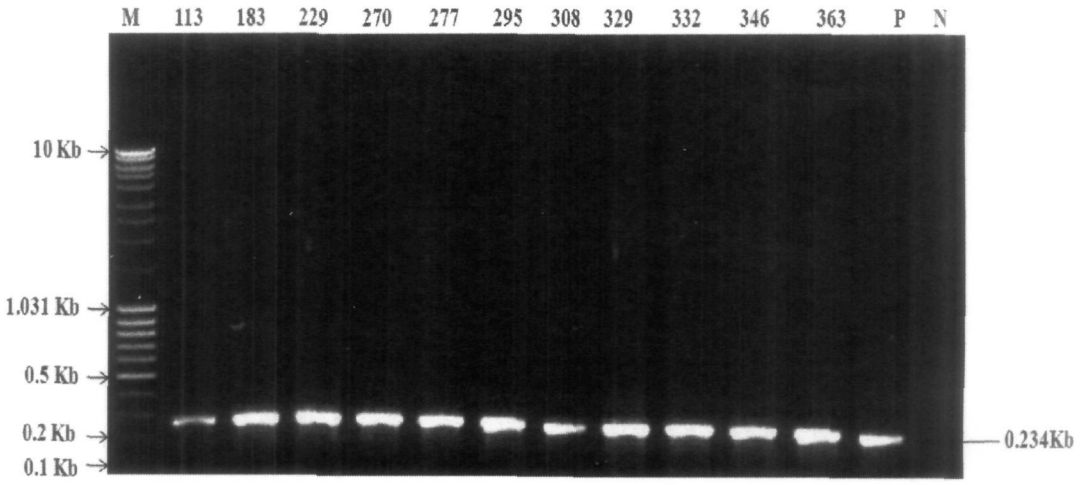


Figure 4.8 PCR amplification of SHV gene, Lane M: 10 Kb DNA ladder, 1-11 *K. penumoniae* clinical isolates (113, 183, 229, 270, 277, 295, 308, 329, 332, 346, 363), Lane P: positive control Lane N: negative control.

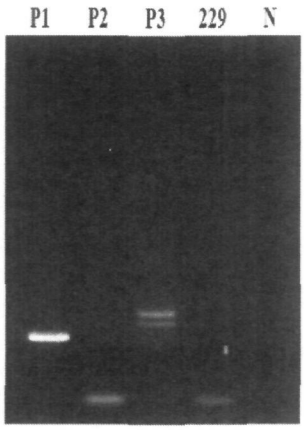


Figure 4.9 PCR amplification of replicon typing, Lane P1 (Inc/HI1), P2 (Inc/HI2), P3 (Inc/I1γ): positive control, 1 *K. penumoniae* clinical isolates (229) and lane N: negative control.

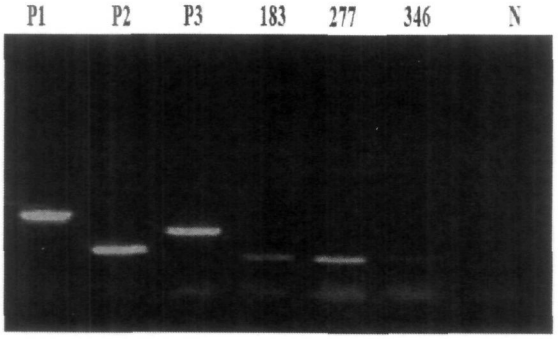


Figure 4.10 PCR amplification of replicon typing, Lane P1 (Inc/L/M), P2 (Inc/X), P3 (Inc/N): positive control, 1-3 *K. penumoniae* clinical isolates (183, 277 and 346) and lane N: negative control.

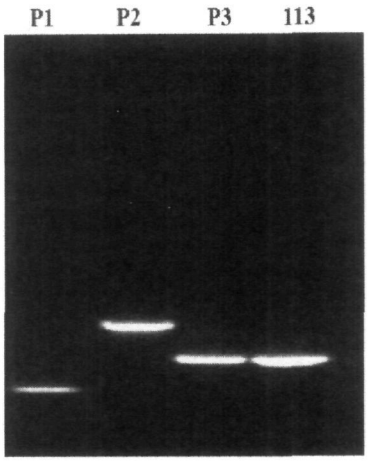


Figure 4.11 PCR amplification of replicon typing, Lane P1 (Inc/Y), P2 (Inc/P), P3 (Inc/FIC): positive control and one *K. penumoniae* clinical isolates (113).

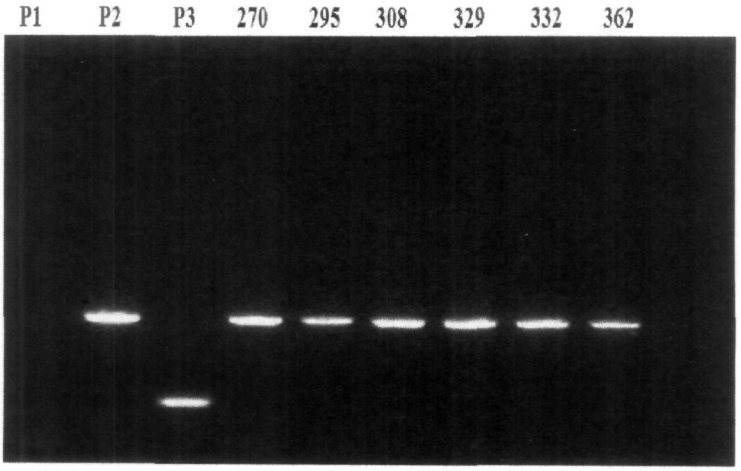


Figure 4.12 PCR amplification of replicon typing, Lane P1 (Inc/FIA), P2 (Inc/FIB), P3 (Inc/W): positive control 1-6 *K. penumoniae* clinical isolates (270, 295, 308, 329, 332 and 363) and lane N: negative control.

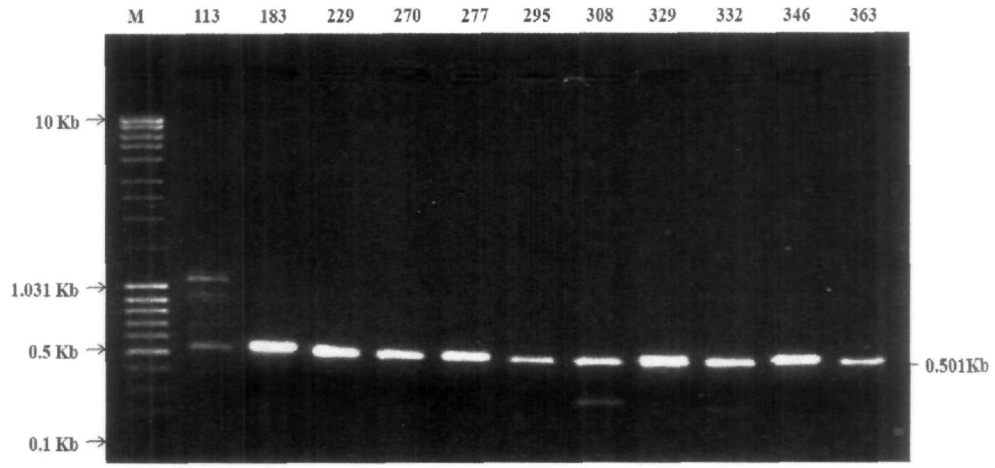


Figure 4.13 PCR amplification of MLST (rpo), Lane M: 10 Kb DNA ladder 1-11 *K. penumoniae* clinical isolates (113, 183, 229, 270, 277, 295, 308, 329, 332, 346, 363).

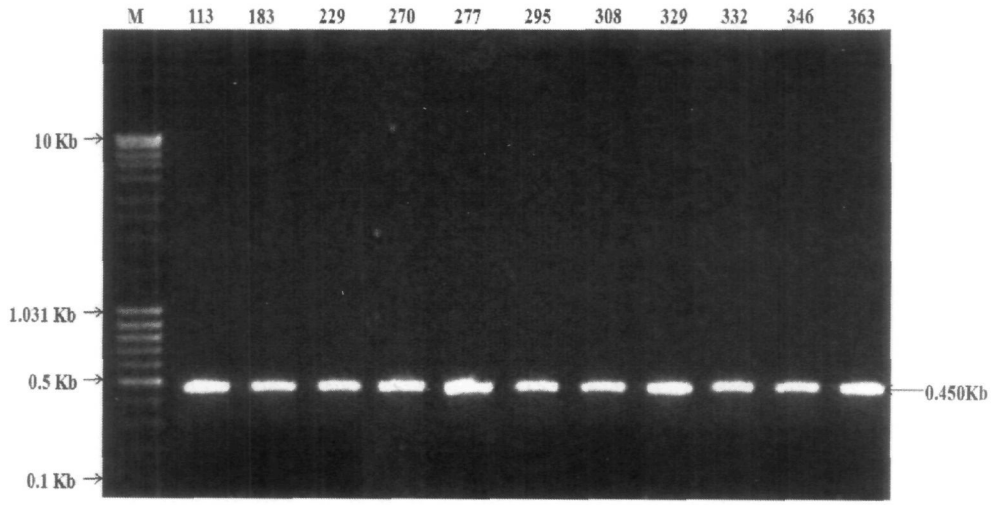


Figure 4.14 PCR amplification of MLST (gap), Lane M: 10 Kb DNA ladder 1-11 *K. penumoniae* clinical isolates (113, 183, 229, 270, 277, 295, 308, 329, 332, 346, 363).

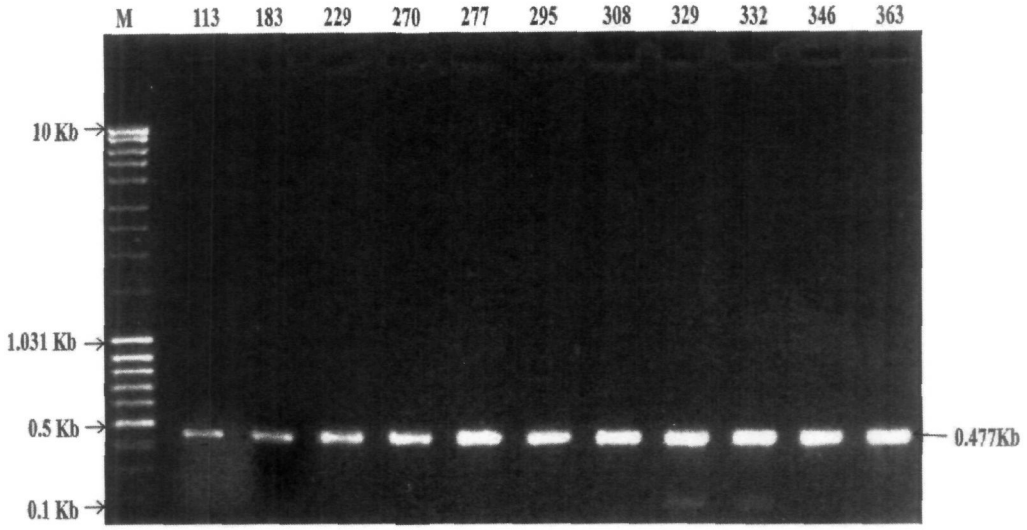


Figure 4.15 PCR amplification of MLST (mdh), Lane M: 10 Kb DNA ladder 1-11 *K. penumoniae* clinical isolates (113, 183, 229, 270, 277, 295, 308, 329, 332, 346, 363).

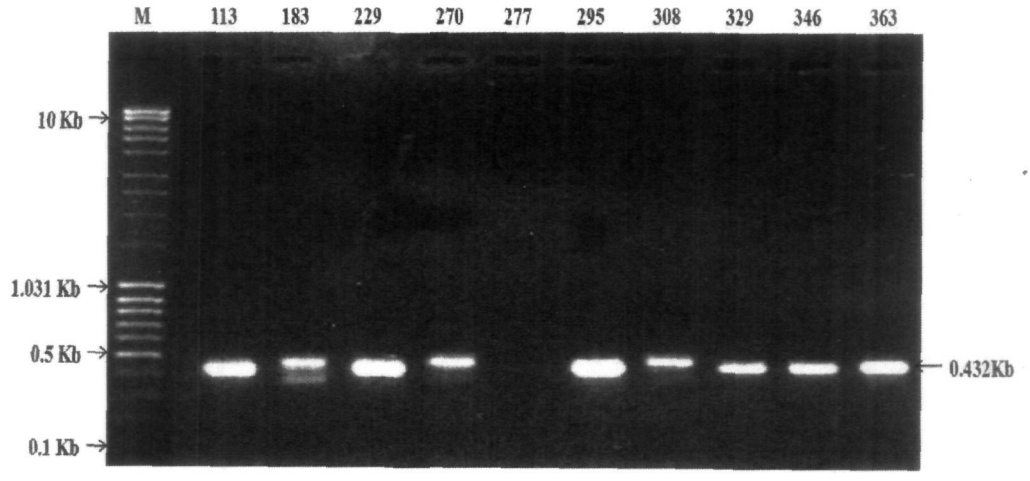


Figure 4.16 PCR amplification of MLST (pgi 2), Lane M: 10 Kb DNA ladder 1-11 *K. penumoniae* clinical isolates (113, 183, 229, 270, 277, 295, 308, 329, 332, 346, 363).

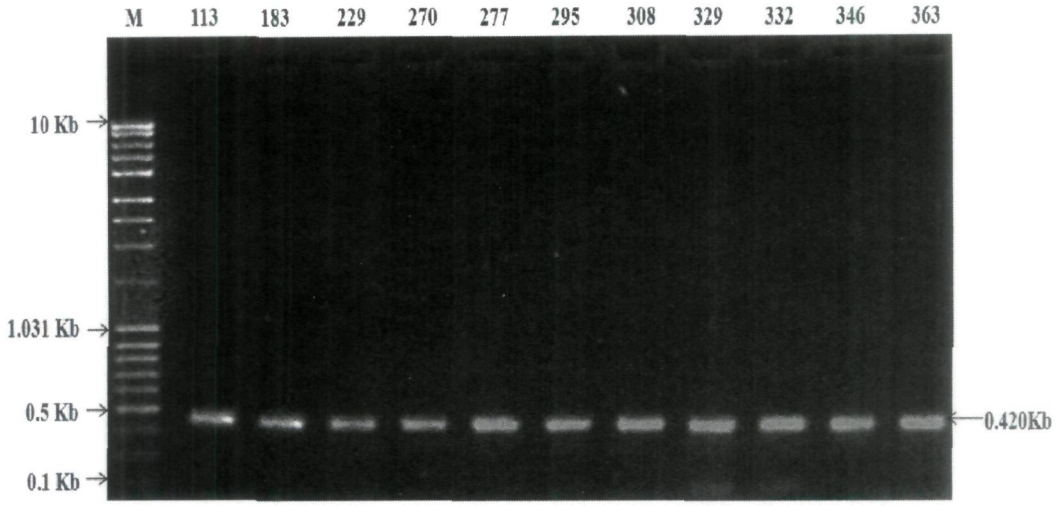


Figure 4.17 PCR amplification of MLST (pho), Lane M: 10 Kb DNA ladder 1-11 *K. penumoniae* clinical isolates (113, 183, 229, 270, 277, 295, 308, 329, 332, 346, 363).

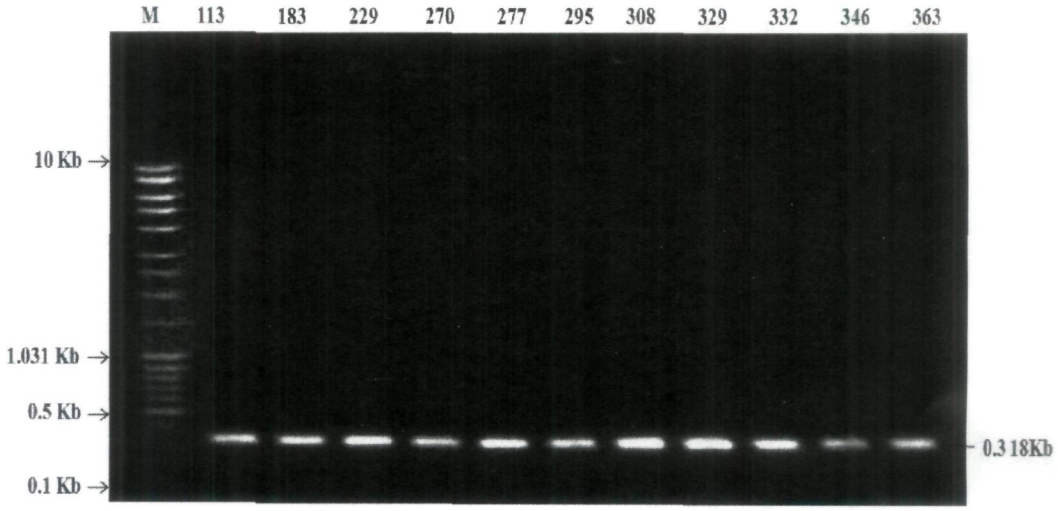


Figure 4.18 PCR amplification of MLST (inf), Lane M: 10 Kb DNA ladder 1-11 *K. penumoniae* clinical isolates (113, 183, 229, 270, 277, 295, 308, 329, 332, 346, 363).

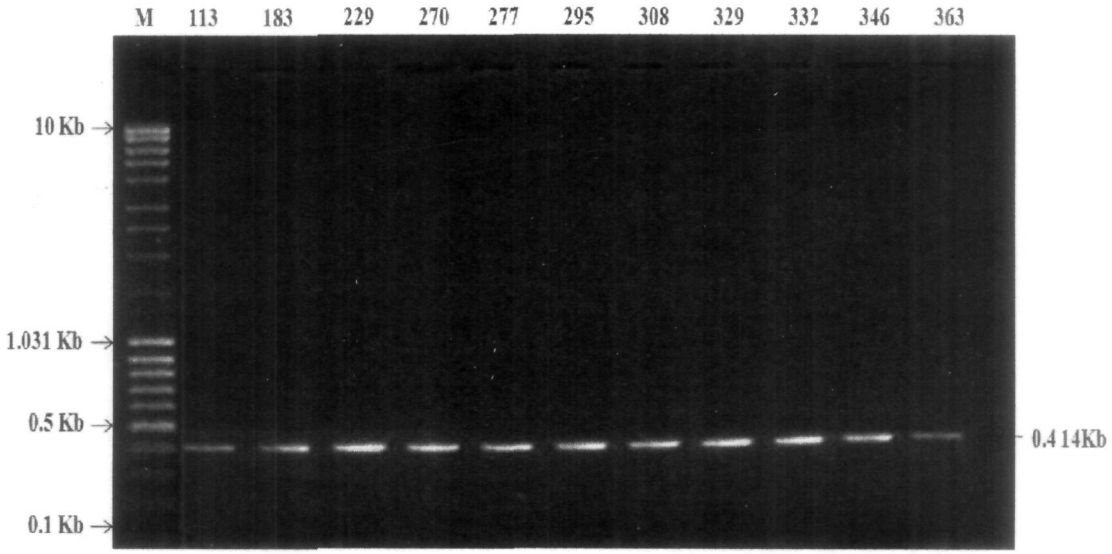


Figure 4.19 PCR amplification of MLST (ton), Lane M: 10 Kb DNA ladder 1-11 *K. penumoniae* clinical isolates (113, 183, 229, 270, 277, 295, 308, 329, 332, 346, 363).

4.4 Discussion

K. pneumoniae is frequently associated with nosocomial infection in NICU and the rate of multi carriage EPK is higher during the neonatal period than in any time of life. The high level of EPK was found among neonates, delivered to mother with lower income groups, illiterate and literate showing highest possibility of acquired neonatal infection (62.13%, 35.92% and 45.63 %) respectively which is an important findings of this study. Our data was consistent with Begum *et al* (2003) which showed education status of mother (OR 2.7) and monthly income of mother belonging to category less than 2000 rupees (OR 5.05), as significant risk factors for preterm neonates [Begum *et al.*, 2003]. Our data was further supported by earlier study which found that the poverty and education are the main risk factor for neonatal infection [Perlinot *et al.*, 2011]. Predisposition to ESBL-producing enterobacteriace carriage in households where the head is unemployed or is an unskilled worker has already been reported in Israel. The highest carriage rates were observed for people with low and intermediate income suggesting that poor sanitary conditions favor the transmission of EPK [Ben-Ami *et al.*, 2006].The possibility of acquiring neonatal infection is 78.64% among preterm and 21.35% among term babies. This is slightly higher than prevailing condition as reported earlier [Riskin *et al.*, 2004]. Preterm babies were found to be a major risk factor for nosocomial infection in previous studies [Hagberg *et al.*, 2005]. However, preterm neonates delivered by mother of Chorio-amnionitics are more prone to have intra-ventricular hemorrhage and there by long-term neurological disability.

Predominance of early onset sepsis and late onset sepsis is 77.66% and 22.33% respectively as shown earlier [Gladstone *et al.*, 1990]. Sepsis is considered to be the result of various risk factors, both maternal and neonatal such as prematurity, low birth weight, hyaline membrane disease, small gestational age, prolonged amniotic membrane rupture of mother and birth defect and asphyxia [Rojo *et al.*, 1999]. No uniform criteria have been established yet for sepsis screening of infants at risk of early onset sepsis.
Among the low birth weight babies, the babies weighing less than 1500 g have the highest possibility of acquiring neonatal infection (60.19%) as compared to the neonates weighing more than 1500 g [Soman *et al.*, 1985]. Low birth weight has been found to be

a great risk factor for nosocomial infection. The main reasons are premature immune systems and extended exposure to broad spectrum antimicrobials in low birth weight neonates. Cell mediated immunity is greatly suppressed in malnourished babies and have a higher incidence of neonatal infection which corroborates with the earlier study [Isaack *et al.*, 1992].

Risk factors for EPK in NICU have been previously identified by univariate analysis. Previously, it was found that duration of hospital stay and early onset sepsis were the independent variable associated with rectal colonization along with EPK [Boo *et al.*, 2005]. Our results partly confirm these findings; total stay in hospital proved to be independent risk factors for acquisition, suggesting that the hospital environment play a crucial role in the transmission of such pathogens. The prevalence of 41.74% neonatal infection was observed in neonates, delivered by mothers having a prolong rupture of membrane (more the 24 hours) as also shown previously [Bizzarro *et al.*, 2005].

These studies revealed the prevalence of *K. pneumoniae* infection in nasal (35.92%), UTI (19.41%) and conjunctivitis (06.70%), the data was comparable to the most recent National Nosocomial Infection Surveillance System (NNISS) data [Gaynes *et al.*, 1996]. This is the first study to the best of our knowledge, conducted in north India, for the multi-carriage infection. It was further observed that prevalence of infection carriage of EPK in the community was not related to age and sex of neonatal infection, but there was significant relation with the isolation of bacteria from medical equipments (like ventilator, radiant wormer, photo therapy, cot, stethoscope, refrigerator and weighing machine) in NICU (29.12%) which is comparable to the NNISS data (26.6%).

EPK might be acquired through horizontal gene transfer. Our data demonstrate that EPK isolates harbored three plasmids (28kb, 64kb and 154kb) and were able to transfer resistance against ceftriaxone. However, varying sized plasmids (5-185 kb) carrying ESBL genes have also been detected in *K. pneumoniae* in earlier studies [Essack *et al.*, 2001]. Our data revealed the presence of $bla_{CTX\text{-}M\text{-}3}$, $bla_{TEM\text{-}1}$ and $bla_{SHV\text{-}1}$ genes on the plasmids which has already been reported earlier in Europe [Livermore *et al.*, 2009]. We have detected 11 class 1 integrons which were invariably PCR positive for *int*-1 and *sul*-1, also demonstrated earlier [Khemtong *et al.*, 2008]. In accordance to our data, hospital dissemination of ESBLs caused by the horizontal transfer of large conjugative plasmids

carrying ESBL producing genes has also been described earlier in both pediatric and adult intensive care unit [Miranda *et al.*, 2004].

In the present study EPK isolate from NICU showed Inc/FIC, Inc/H12, Inc/X and Inc/FIB replicon types of plasmids (Table 4). This has already been shown earlier in which plasmids of Inc/HI2 and Inc/FIB were associated with the ***bla***$_{CTX-M-3}$ and ***bla***$_{TEM-1}$ gene deriving from *K. pneumoniae* [Carattoli *et al.*, 2009].

MLST was performed on the basis of ERIC typing and incompatibility and was found ST-64 (KP113), ST-280 (KP277) and ST-51 (KP229) as shown in table 4. ST-520 is genetically close variant of ST-64; ST 520 is a single locus variant (rpo allele), ST-131 is genetically close variant of ST-51; ST-131 is a single locus variant (pho allele) and ST-65 is genetically close variant of ST-280; ST-65 is also a single locus variant (ton alleles) of ST 280. Two clinical isolates (KP113, KP229) which were from the different patients and one of them was environmental isolate (KP277) in NICU (Table 4). All four EPK STs described in the present study indicates that the most of the strains are scattered throughout the phylogenetic tree of *K. pneumoniae* rather than clustered into a single genetic lineage which suggest that dissemination of EPK resistance is due to horizontal gene transfer [Son *et al.*, 2010].

One of the most striking findings in the present study was the high level of resistance to third generation cephalosporins among *K. pneumoniae* isolates. Almost 68% isolates were resistant to cefotaxime, ceftriaxone and ceftazidime. The SENTRY surveillance program reported the frequency of EPK to be approximately 37% in Latin America and 7% in the United States [Sader *et al.*, 1998]. Within the Asian Pacific region, the prevalence of EPK was reported to be 5%, 21.7%, 31% and 38% in Japan, Taiwan, Philippines and Malaysia/Singapore, respectively. The present data show resistance against multiple group of antibiotic (β-lactam, aminoglycosides, fluoroquinolone and tetracycline). This is consistent with previous findings [Bizzarro *et al.*, 2007]. In the present study, *K. pneumoniae* isolates were found to be highly resistant to tetracycline (78.64%) and cotrimoxazole (91.26%). This is probably due to the fact that this antibiotic has been widely used over the past decade in this region because of the low cost and easy availability to the poor people residing in various underdeveloped pockets of the

otherwise developing nation. Similar studies have also been performed in other parts of India. Our data have shared harmony to previous reports [Shobha *et al.*, 2007].

4.5 Conclusions

The study concludes that the independent risk factors for EPK infection are low socioeconomic status and illiteracy of mother. Moreover, the use of carbapenem, Ceftazidime, gatifloxacine, ciprofloxacin and amikacin may enhance the susceptibility of EPK. In order to prevent the EPK infection in neonates, avoiding non judicial use of antibiotics and common sharing of the unsterilized equipments should not be practiced.

Chapter-5

Molecular mechanism of inhibitor resistant ESBLs producing K. pneumoniae isolates from NICU

5.1 Introduction

Multidrug resistant gram negative bacteria are a major therapeutic challenge both in NICU and hospital settings [Chiu *et al.*, 2000]. These isolates are getting resistant to multiple antimicrobial agents, including aminoglycosides, quinolones, extended spectrum cephalosporins by hydrolysis of the β-lactam ring and are inhibited by β-lactamase inhibitors. There are more than 400 extended spectrum β-lactamases (ESBLs) described so far, mostly derived from the groups CTX-M, TEM, SHV and OXA [Coque *et al.*, 2008]. However, these acquisition rates increase dramatically in hospitalized patients in direct proportion to the length of stay. Moreover, *K. pneumoniae* is very well adapted to the hospital environment since it exhibits higher survivability on hands and environmental surfaces than other member of *Enterobacteriaceae* [Haque *et al.*, 2011]. Such infections mainly concern with the patients admitted to NICU with several co-morbidities and a history of prolonged administration of antibiotics [Pena *et al.*, 2007]. On the other hand, simultaneous production of OXA-1 β-lactamase is poorly inhibited by clavulanic acid, tazobactam or sulbactam and has negligible activity against first-generation cephalosporins [Poirel *et al.*, 2004]. Antibiotic selection for such infective strains thus becomes a therapeutic challenge. Outbreaks by ESBL producing organisms have been reported frequently in NICU settings [Mirelis *et al.*, 2003]. Clinical conditions and treatments predisposing to infection by such pathogens have been investigated but very few studies were able to identify the prevalence of *K. pneumoniae* producing ESBLs in NICU and there is a paucity of ESBL data concerning neonates, particularly in India. In view of the present situation, we initiated our study to understand the mode of mechanism among the *K. pneumoniae* isolates, circulating in NICU of a teaching hospital of North India.

5.2 Experimental outline

The present study was previously described in section 2.2.1. Antimicrobial susceptibility testing of the isolates was performed by the CLSI standard disc diffusion method as outlined in section 2.2.2. The ESBL and MBL phenotypic confirmatory test was performed as mentioned in section 2.2.2. MICs of monobactam, cephalothin, cefazolin,

cefuroxime, cephoxitine, ceftazidime, cephotaxime, cefepime, imipenem, ertapenem, meropenem and aztreonam were determined by the CLSI microbroth dilution method.

A search for bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{OXA-1}, bla_{KPC}, bla_{NDM-1}, ArmA and RmtA genes and integrons were carried out using oligonucleotide specific primers as listed in table 2.1 for both genomic and plasmid DNA. Molecular typing was performed by ERIC-PCR, as outlined in section 2.2.7. PCR-based replicon typing was carried out on 15 non-conjugative plasmids as described in chapter 4.

5.3 Results

A total of 3000 neonates that were screened for the nosocomial infection in the NICU, 785 (26.16%) were found to have clinically suspected culture. In which, antibiotic susceptibility test was performed to detect 262 (33.37 %) ESBL producing *K. pneumoniae* isolates.

Antibiotic susceptibility test were performed for all ESBL producing *K. pneumonia* isolates (Table 5.1). ESBL positive isolates of *K. pneumoniae* were found to have significantly higher resistance against these antibiotics. Our study showed that among the β-lactum groups, higher percent of resistant strains were observed against penicillin (95.80%), followed by pipracillin (91.22%). Highest resistance was observed against cephalothin and cephazolin in first generation of cephalosporin group. The pattern of resistance among the second generation of cephalosporins that is cefuroxime and cephoxitin, were 207 (79.00%) against cefuroxime and 199 (75.95%) against cephoxitin. Resistant strains against ceftriaxone were found to be 171 (65.26%). Moreover, in the present study it was found that, 86.78% *K. pneumoniae* strains showed resistance against aztreonam. A total of 78 (29.77%) ESBL producing *K. pneumoniae* strains were found resistant against cefepime (Table 5.1). Highest percent resistance was observed against gentamicin (74.80%) whereas, 43.12% *K.pneumoniae* strains were found to be resistant against amikacin (Table 5.1). Among the resistance against fluoroquinolones, highest percentage of resistance was observed against nalidixic acid (80.53%), while the resistance against gatifloxacin was found to be 38.54%. The susceptibility of these isolates was also investigated against cotrimoxazol, tetracycline and oxacillin and they showed

higher percentage of resistance (Table 5.1). Moreover, the resistance against amoxyclav, ampicillin/sulbactam,amoxicillin/sulbactam,cefixime/clavulanicacid,ceftazidim/clavulanic acid, ceftriaxon/sulbactam, ceftriaxon/tazobactam and cefepime/tazobactam was found to be 92.74%, 86.78%, 72.13%, 59.92%, 53.43%, 52.67%, 49.61% and 49.61% respectively. In addition, some of the ESBL producing *K. pneumoniae* isolates from neonates shared resistance against meropenem (Table5.1). ESBL producing *K. pneumoniae* strains isolated from NICU, found to have multiple plasmids of different molecular size (Table 5.2). Among 262 ESBL producing *K. pneumoniae* isolates with two or three plasmids, the presence of small size transferable plasmids (28 kb) was detected in 16 (6.10%) ESBL producing *K. pneumoniae* isolates. 246 (93.89%) of them harbored a conjugative mega plasmid. These mega plasmids bear gene imparting resistance against extended spectrum cephalosporins. The transconjugants were also tested for their susceptibility against various antibiotics of β-lactam, aminoglycoside and fluroquinolone group using disk diffusion test and microdilution method. The tranconjugants showed multidrug resistance (MDR) phenotype including resistance to all generation of cephalosporine.

ERIC-PCR was performed for the molecular characterization of 262 strains of *K. pneumoniae*, which categorized the strains in 15 different phylogenic groups (Figure5.1). One strain from each phylogenic group was further confirmed by ERIC-PCR as shown in figure 5.2. The Ad group was most frequently observed among the isolates (78 of 262 strains, 29.77%) as shown in figure 5.1. The types of ESBL producing *K. pneumoniae* are summarized in table 5.2. Of 262 *K. pneumoniae* cases 15 had $bla_{\text{CTX-M-3}}$, 13 had $bla_{\text{TEM-1}}$, 10 had $bla_{\text{SHV-1}}$, 15 had $bla_{\text{OXA-1}}$, 9 had Arm-A and 7 were found to have Rmt-A. Integrons were also detected in all 15 strains with a consistent size of 530bp (Figure 5.3), representing each phylogenic group and was found as class 1 integrons carrying *Sul*-1 (Figure 5.4) and *Int*-1genes (Table 5.2, Figure 5.5). None of them was amplified for bla_{KPC}, $bla_{\text{NDM-1}}$. Amplified products for each $bla_{\text{CTX-M}}$ (Figure 5.6), bla_{TEM} (Figure 5.7), bla_{SHV} (Figure 5.8), $bla_{\text{OXA-1}}$ (Figure 5.9), Arm-A (Figure 5.10) and Rmt-A (Figure 5.11) were sequenced and analyzed using NCBI data base site (http/www.ncbi.nlm.nih.gov/) replicon types (Table 5.2).

Table 5.1 Representation of antibiogram

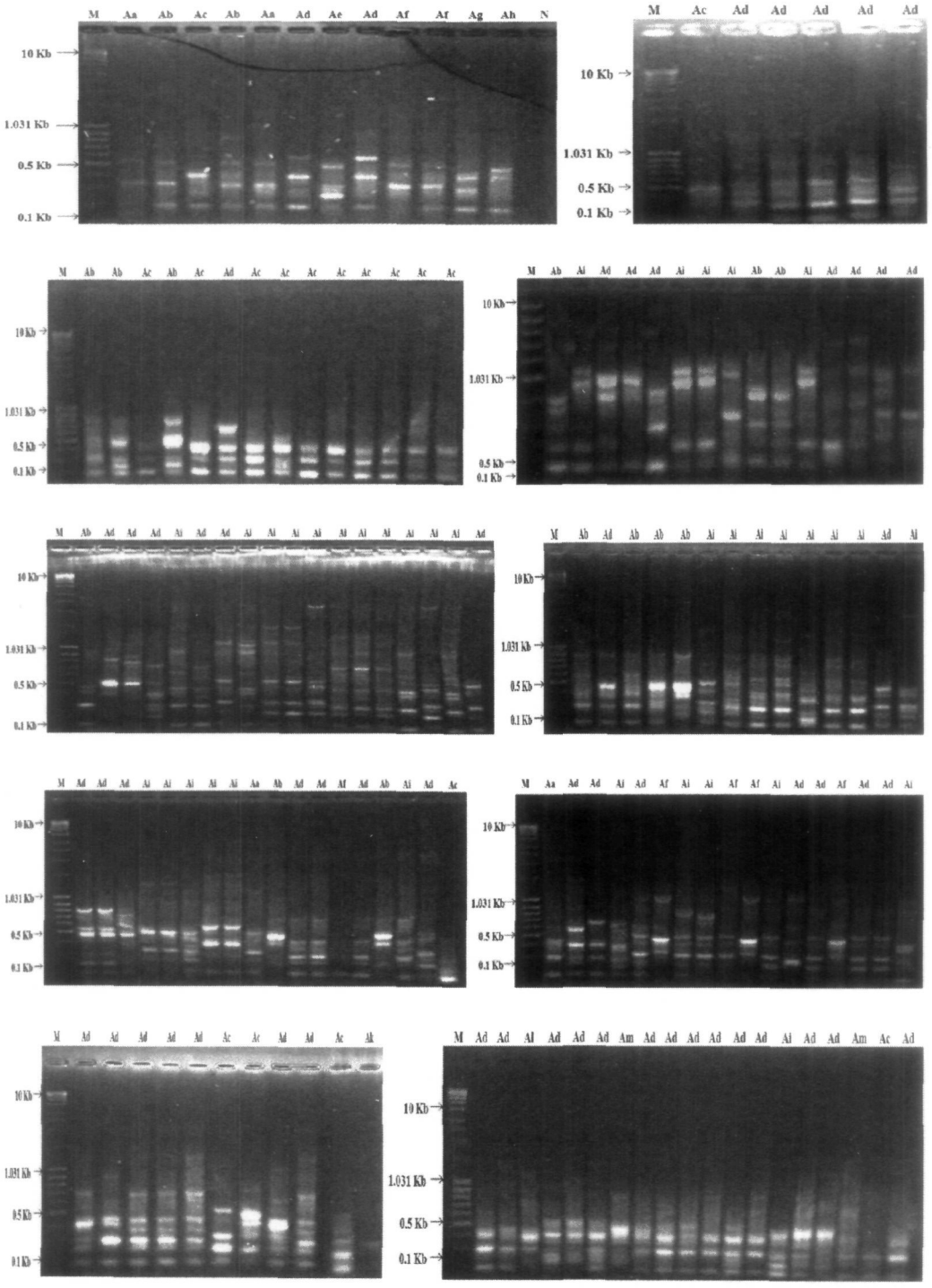
Antibiotic groups		Antibiotics	% Resistance to antibiotics N=262 (71.78%)
Aminoglycosides		G	196(74.80)
		Tb	130(49.61)
		Ak	113(43.12)
Fluoroquinolones		Na	211(80.53)
		Cf	157(59.92)
		Gf	101(38.54)
β-Lactams		A	243(92.74)
		P	251(95.80)
		Pc	239(91.22)
Others		T	190(72.51)
		Co	223(85.11)
		Ox	190(72.51)
		Ch	230(87.78)
Cephalos porins	1^{st}g	Cz	227(86.64)
		Cu	207(79.00)
	2^{n}g	Cn	199(75.95)
		Ca	189(72.13)
	3^{rd}g	Ce	178(67.93)
		Ci	171(65.26)
	4^{th}g	Cpm	78(29.77)
Carbapenems		Imp	00(00.00)
		Ert	00(00.00)
		Mr	22(08.39)
Monobactam		Ao	227(86.78)
Inhibitor		Ac	243(92.74)
Antibiotics		As	227(86.78)
combination with		Ams	189(72.13)
Inhibitors		Cmc	157(59.92)
		Cac	140(53.43)
		Cis	138(52.67)
		Cit	130(49.61)
		Cpt	130(49.61)

Generation (g), Gentamicin (G), Tobramycin (Tb), Amikacin (Ak), Nalidixicacid (Na), Ciprofloxacin (Cf), Gatifloxacin (Gf), Ampicillin (A), Penicillin (P), Pipracillin (Pc), Tetracyclin (T), Co-trimoxazole (Co), Oxacillin (Ox), Cephalothin (Ch), Cefazolin (Cz), Cefuroxime (Cu), Cephoxitin (Cn), Ceftazidime (Ca), Cephotaxime (Ce), Ceftriaxon (Ci), Cefepime (Cpm), Imipenem (Imp), Ertapenem (Ert), Meropenem (Mr), Aztreonam (Ao), Amoxyclav (Ac), Ampicillin/Sulbactam (As), Amoxicillin/Sulbactam (Ams), Cefixime/Clavulanic acid (Cmc), Ceftazidim/Clavulanicacid (Cac), Ceftriaxon/Sulbactam (Cis), Ceftriaxon/Tazobactm (Cit),Cefepime/Tazobactam (Cpt).

Table 5.2 MICs of β-lactams and genetic analysis of resistant markers in plasmids

S.no	Number of Plasmid and size(Kb)	Resistance Marker	MIC(µg/ml)												Transconjugants	Class of Integrons	Plasmid Incompatibility groups
			Cephalosporins								Carbapenems			Mb	Resistance Marker Transfered		
			Ch	Cz	Cu	Cn	Ca	Ce	Cl	Cpm	Imp	Ert	Mr	Ao			
Kp-19	154,64,28	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA-1	31.25	15.62	62.50	15.62	31.25	31.25	07.81	07.81	02	02	02	250	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA-1	1	I1γ
Kp-30	154,64,28	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA-1, *bla*SHV-1,Arm-A, rmt-A	15.62	31.25	15.62	31.25	31.25	62.50	07.81	07.81	02	02	262.50	500	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA1, *bla*SHV-1, Arm-A, rmt-A	1	FIA/FIB
Kp-55	154,64,28	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA-1, *bla*SHV-1,Arm-A, rmt-A	31.25	15.62	31.25	15.62	31.25	62.50	07.81	07.81	02	02	02	500	*bla*CTX-M,*bla*TEM,*bla*OXA1, *bla*SHV-1, Arm-A, rmt-A	1	FIA/FIB
Kp-108	154,64,28	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA-1, *bla*SHV-1,Arm-A	15.62	31.25	31.25	15.62	31.25	62.50	07.81	07.81	02	02	02	500	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA1, *bla*SHV-1, Arm-A	1	FIB
Kp-140	154,64,28	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA-1, Arm-A, rmt-A	15.62	15.62	31.25	31.25	31.25	62.50	07.81	07.81	02	02	02	500	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA1, Arm-A, rmt-A	1	FIA/FIB
Kp-160	154,64,28	*bla*CTX-M-3, *bla*SHV-1, *bla*OXA-1, Arm-A	31.25	15.62	31.25	15.62	31.25	62.50	07.81	07.81	02	02	02	500	*bla*CTX-M-3,*bla*OXA-1,*bla*SHV-1, Arm-A	1	FIB
Kp-161	154,64,28	*bla*CTX-M-3,*bla*OXA-1,	31.25	15.62	31.25	15.62	31.25	62.50	07.81	07.81	02	02	02	500	*bla*CTX-M-3,*bla*OXA1	1	I1γ
Kp-182	154,64,28	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA-1, *bla*SHV-1,Arm-A,rmtA	31.25	15.62	31.25	15.62	31.25	62.50	07.81	07.81	02	02	02	500	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA1, *bla*SHV-1, Arm-A,rmtA	1	HI2
Kp-207	154,64,28	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA-1,	31.25	15.62	31.25	15.62	31.25	62.50	07.81	07.81	02	02	02	500	*bla*CTX-M,*bla*TEM,*bla*OXA1	1	I1γ
Kp-240	154,64,28	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA-1,	31.25	15.62	31.25	15.62	31.25	62.50	07.81	07.81	02	02	02	250	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA1	1	I1γ
Kp-286	154,64,28	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA-1, *bla*SHV-1,Arm-A	31.25	15.62	31.25	15.62	31.25	62.50	07.81	07.81	02	02	02	500	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA1, *bla*SHV-1, Arm-A	1	HI1
Kp-290	154,64,28	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA-1, *bla*SHV-1,Arm-A, rmt-A	31.25	15.62	31.25	15.62	31.25	62.50	07.81	07.81	02	02	02	500	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA1, *bla*SHV-1, Arm-A, rmt-A	1	FIA/FIB
Kp-296	154,64,28	*bla*CTX-M-3,*bla*TEM-1, *bla*SHV-1, *bla*OXA-1,	31.25	15.62	31.25	15.62	31.25	62.50	07.81	07.81	02	02	02	500	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA1,	1	FIA/FIB
Kp-367	154,64,28	*bla*CTX-M,*bla*TEM,*bla*OXA-1, *bla*SHV	31.25	15.62	31.25	15.62	31.25	62.50	07.81	07.81	02	02	02	500	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA1, *bla*SHV-1	1	I1γ
Kp-375	154,64,28	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA-1, *bla*SHV-1,Arm-A, rmt-A	31.25	15.62	31.25	15.62	31.25	62.50	07.81	07.81	02	02	02	500	*bla*CTX-M-3,*bla*TEM-1,*bla*OXA1, *bla*SHV-1, Arm-A, rmt-A	1	I1γ

Monobactam (Mb), Cephalothin (Ch), Cefazolin (Cz), Cefuroxime (Cu), Cephoxitin (Cn), Ceftazidime (Ca), Cephotaxime (Ce), Cefepime (Cpm), Imipenem (Imp), Ertapenem (Ert), Meropenem (Mr), Aztreonam (Ao)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1.031 Kb
0.5 Kb
0.1 Kb
M Ac Ad Ad Ad Ad Ad
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1.031 Kb
0.5 Kb
0.1 Kb
M Ab Ab Ac Ah Ac Ad Ac Ac Ac Ac Ac Ac Ac
10 Kb
1.031 Kb
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10 Kb
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0.1 Kb

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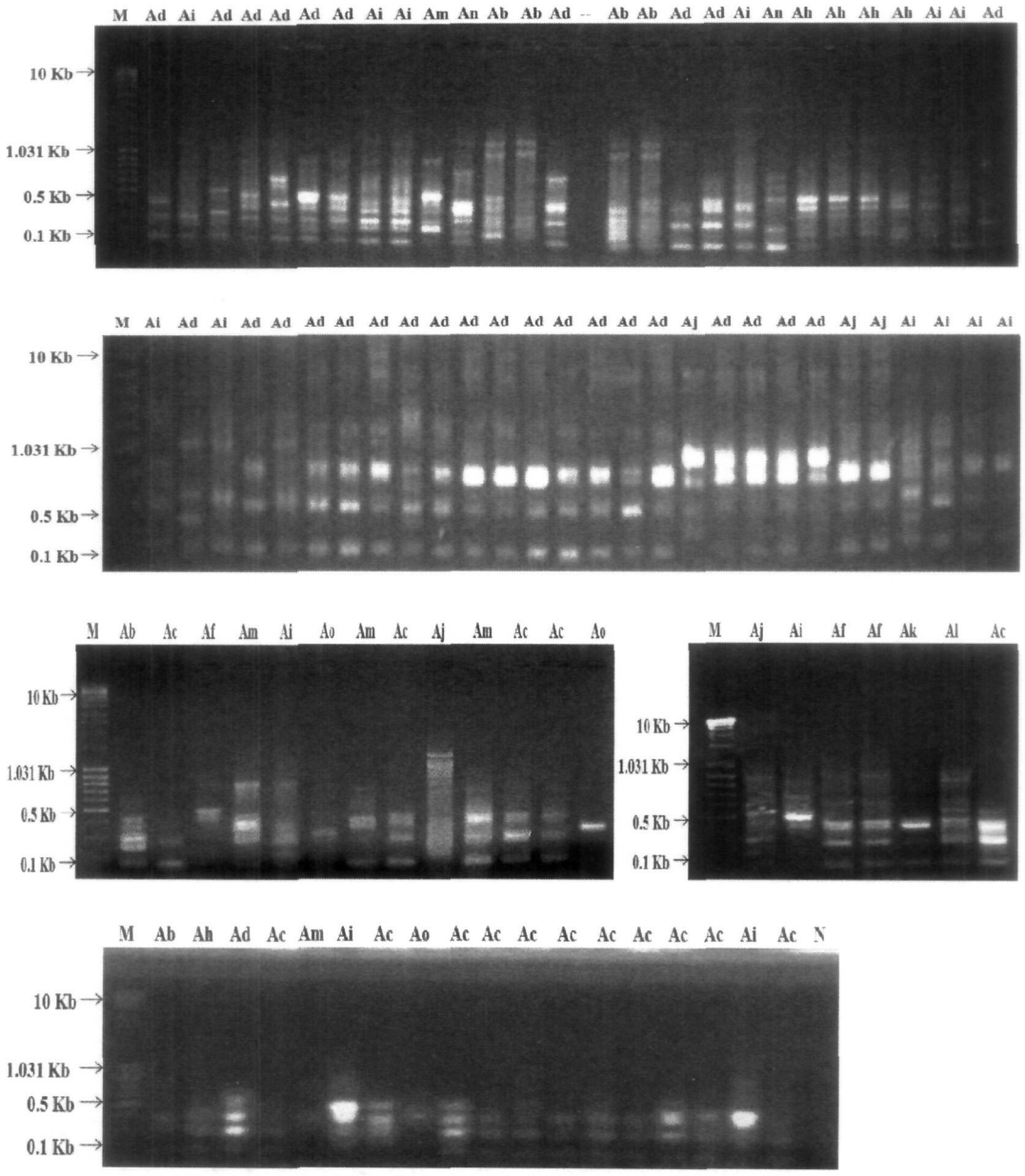

Figure 5.1 ERIC profiles of ESBL producing *K. pneumoniae* isolates from NICU. M stands for marker (the first lane of each gel), Aa-Ao represents clinical isolates and N stands for negative control (the last lane of first and last gel).

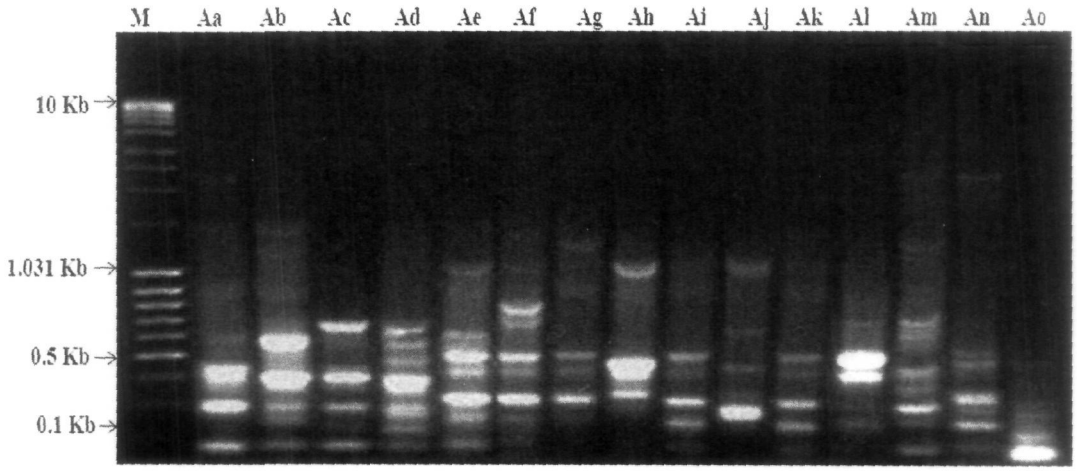

Figure 5.2 ERIC profile for reconfirmation of 15 different phylogenic groups of ESBL producing *K. penumoniae* in NICU. M stands for marker (the first lane of gel), Aa-Ao represents clinical isolates.

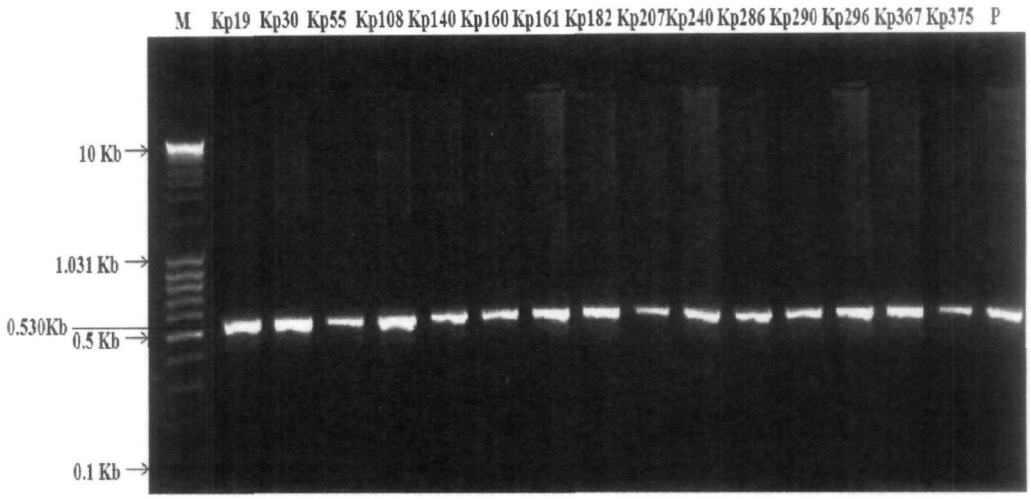

Figure 5.3 PCR amplification of 5'-CS-3'CS conserved sequences of integron. Lane M: 10 Kb DNA ladder, 1-15 *K. penumoniae* clinical isolates (Kp19, Kp30, Kp55, Kp108, Kp140, Kp160, Kp161, Kp182, Kp207, Kp240, Kp286, Kp90, Kp296, Kp367, Kp375) and Lane P: positive control.

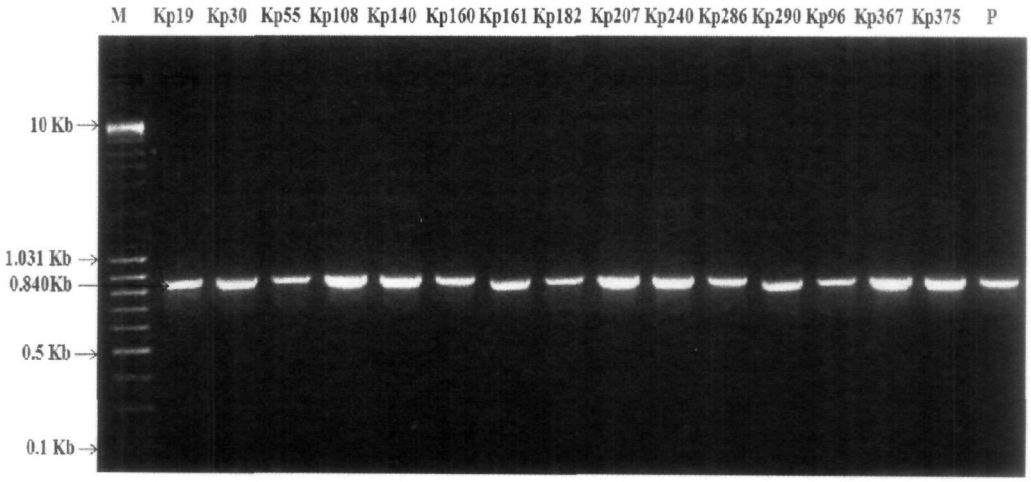

Figure 5.4 PCR Integron mapping by PCR amplification of Sul-1 gene. Lane M: 10 Kb DNA ladder, 1-15 *K. penumoniae* clinical isolates (Kp19, Kp30, Kp55, Kp108, Kp140, Kp160, Kp161, Kp182, Kp207, Kp240, Kp286, Kp290, Kp296, Kp367, Kp375) and Lane P: positive control.

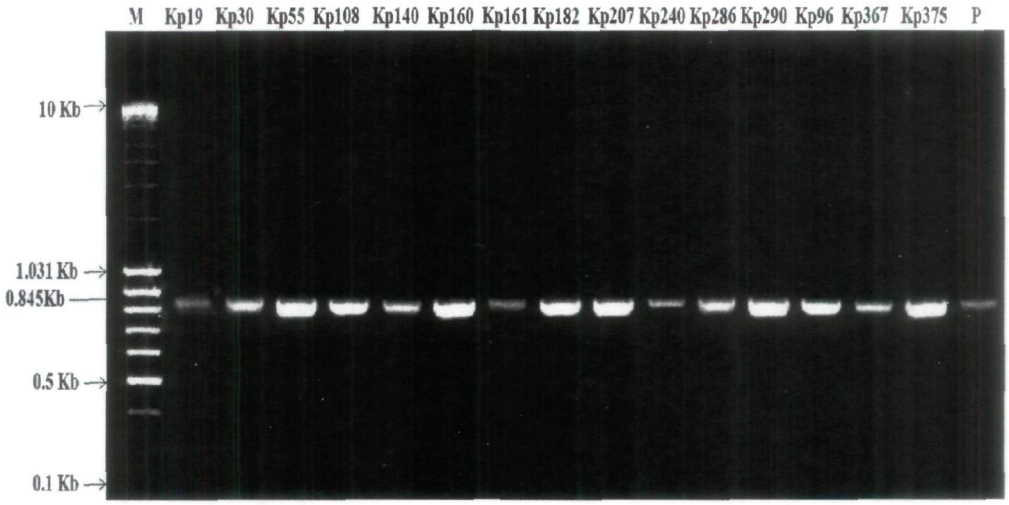

Figure 5.5 Integron mapping by PCR amplification of Int-1 gene. Lane M: 10 Kb DNA ladder, 1-15 *K. penumoniae* clinical isolates (Kp19, Kp30, Kp55, Kp108, Kp140, Kp160, Kp161, Kp182, Kp 207, Kp240, Kp286, Kp290, Kp296, Kp367, Kp375) and Lane P: positive control.

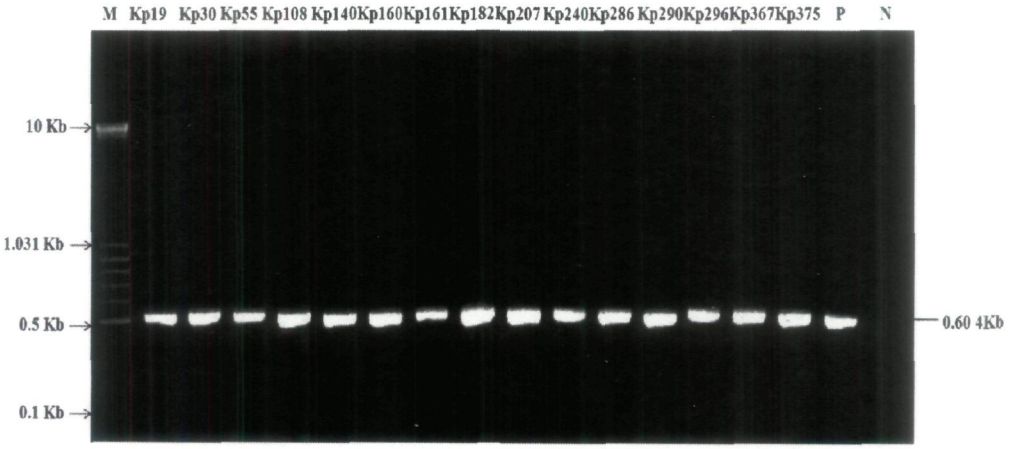

Figure 5.6 PCR amplification of CTX-M gene, Lane M: 10 Kb DNA ladder, 1-15 *K. penumoniae* clinical isolates (Kp19, Kp30, Kp55, Kp108, Kp140, Kp160, Kp161, Kp182, Kp 207, Kp 240, Kp 286, Kp290, Kp296, Kp367, Kp375), Lane P: positive control Lane N: negative control.

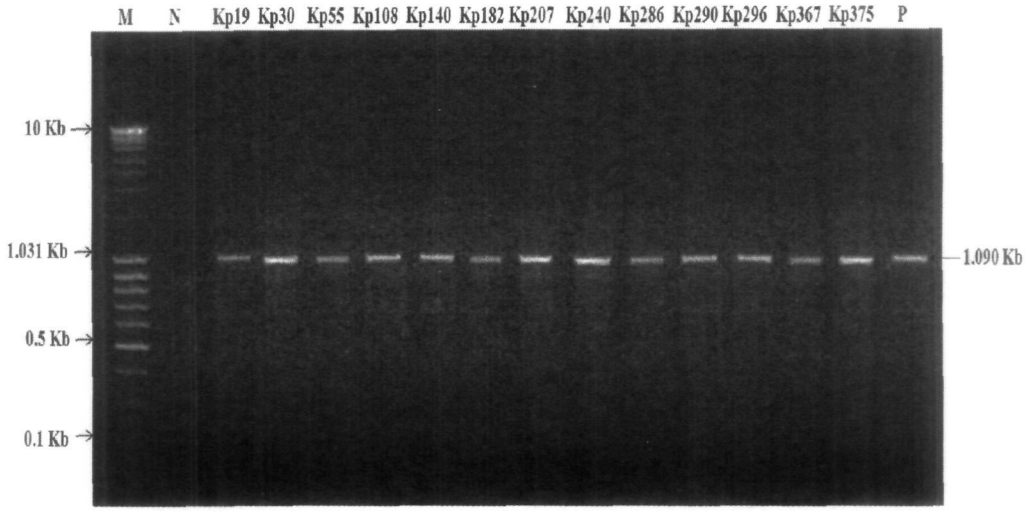

Figure 5.7 PCR amplification of TEM gene, Lane M: 10 Kb DNA ladder, Lane N: negative control 1-14 *K. penumoniae* clinical isolates (Kp19, Kp30, Kp55, Kp108, Kp140, Kp182, Kp207, Kp240, Kp 286, Kp290, Kp296, Kp367, Kp375), Lane P: positive control.

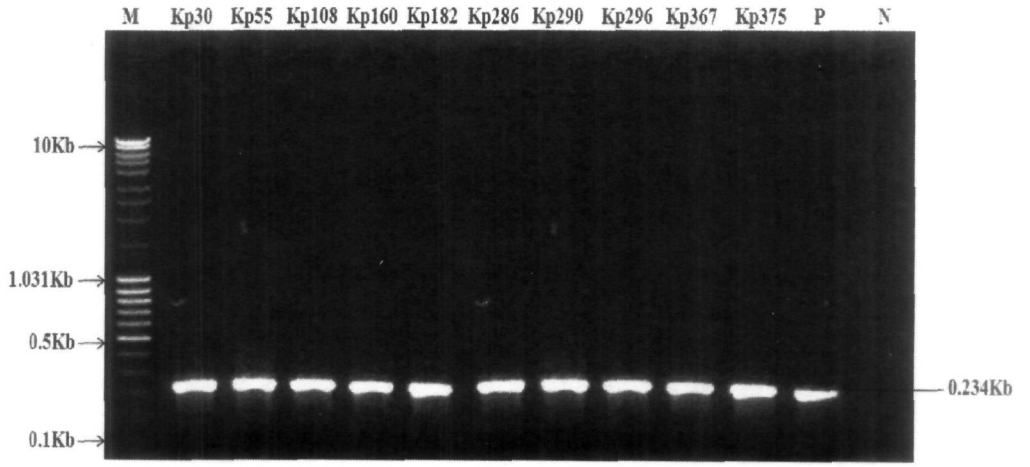

Figure 5.8 PCR amplification of SHV gene, Lane M: 10 Kb DNA ladder, Lane N: negative control 1-14 *K. penumoniae* clinical isolates (Kp30, Kp55, Kp108, Kp160, Kp182, Kp286, Kp 290, Kp 296, Kp 367, Kp 375), Lane P: positive control and Lane N: negative control.

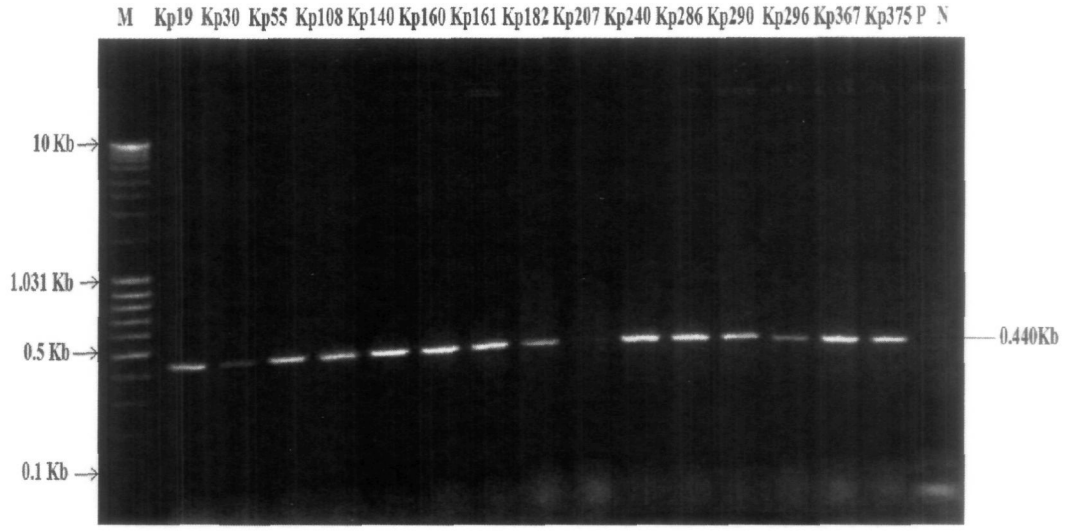

Figure 5.9 PCR amplification of OXA-1 gene, Lane M: 10 Kb DNA ladder, 1-15 *K. penumoniae* clinical isolates (Kp19, Kp30, Kp55, Kp 108, Kp140, Kp160, Kp161, Kp182, Kp 207, Kp 240, Kp 286, Kp 290, Kp 296, Kp 367, Kp 375), Lane P: positive control Lane N: negative control.

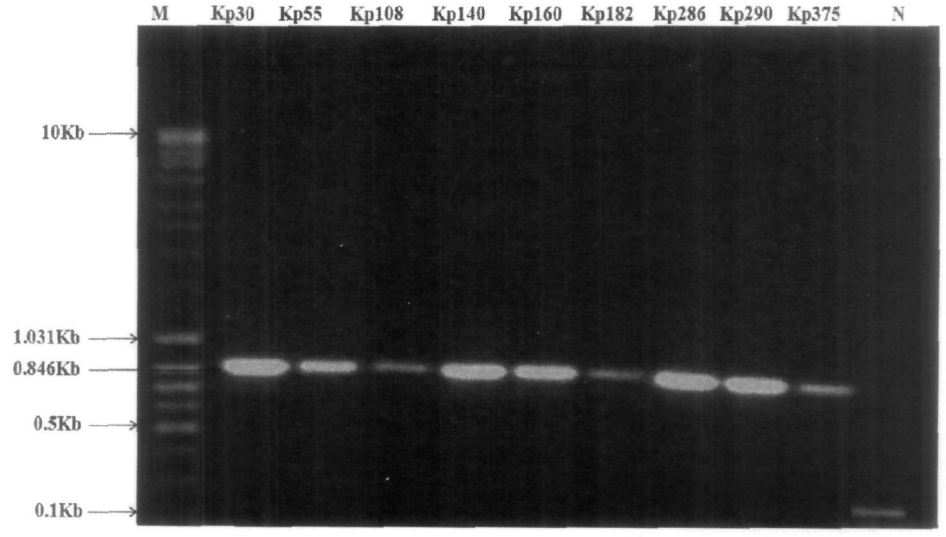

Figure 5.10 PCR amplification of Arm-A gene, Lane M: 10 Kb DNA ladder, 1-9 *K. penumoniae* clinical isolates (Kp30, Kp55, Kp 108, Kp140, Kp160, Kp182, Kp 286, Kp 290, Kp 375), Lane N: negative control.

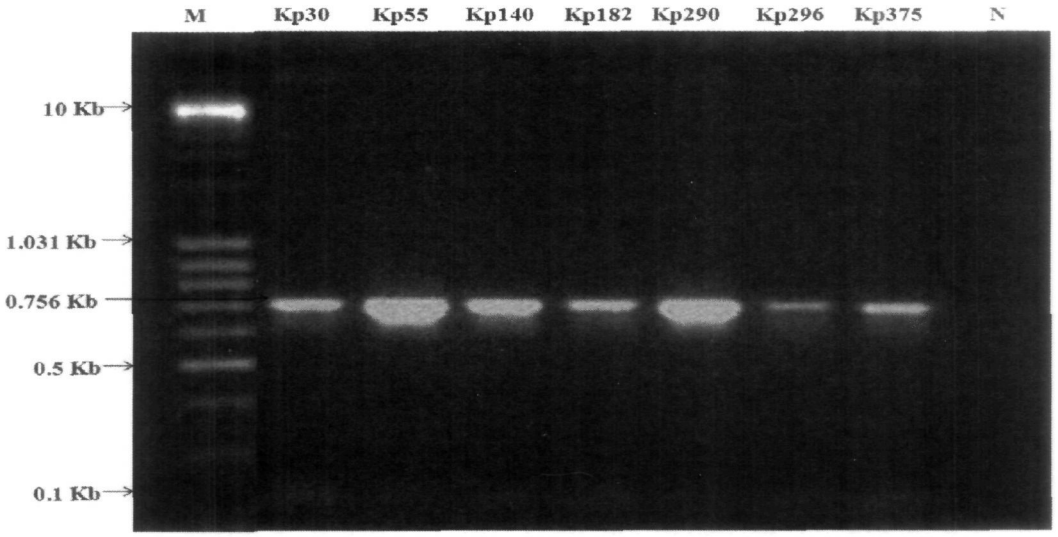

Figure 5.11 PCR amplification of Rmt-A gene, Lane M: 10 Kb DNA ladder, 1-7 *K. penumoniae* clinical isolates (Kp30, Kp55, Kp140, Kp182, Kp290, Kp296, Kp375), Lane N: negative control.

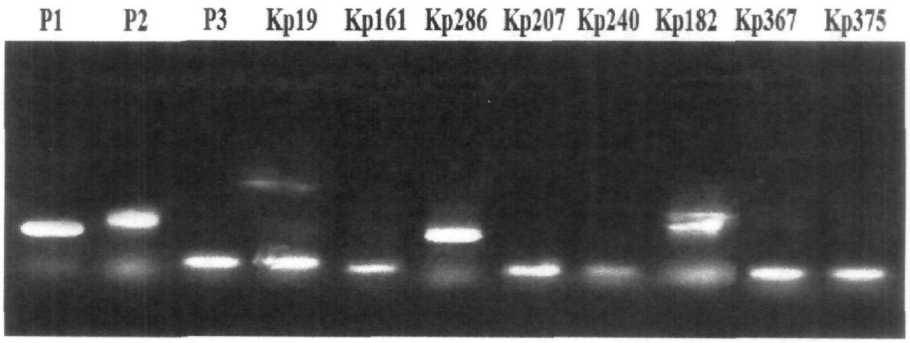

Figure 5.12 PCR amplification of replicon typing, Lane P1 (Inc/HI1), P2 (Inc/HI2), P3 (Inc/I1γ): positive control, 1-8 *K. penumoniae* clinical isolates (Kp19, Kp161, Kp286, Kp207, Kp240, Kp182, Kp367, Kp375).

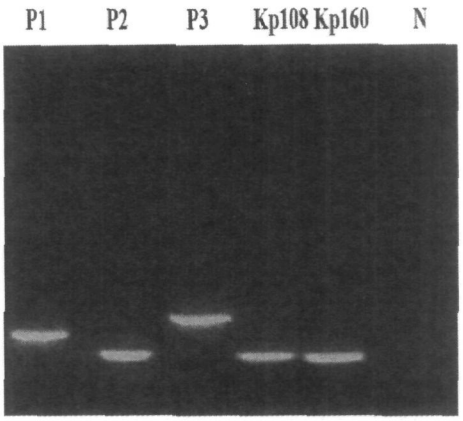

Figure 5.13 PCR amplification of replicon typing, Lane P1 (Inc/FIA), P2 (Inc/FIB), P3 (Inc/W): positive control, 1-2 *K. penumoniae* clinical isolates (Kp108, Kp160) Lane N: negative control.

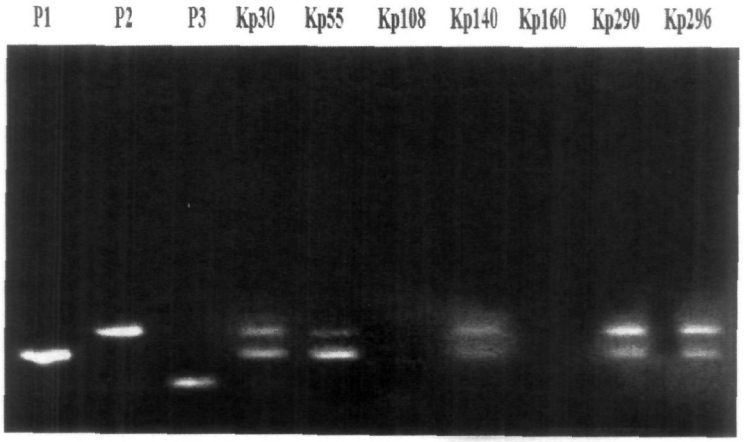

Figure 5.14 PCR amplification of replicon typing, Lane P1 (Inc/FIA), P2 (Inc/FIB), P3 (Inc/W): positive control, 1-7 *K. penumoniae* clinical isolates (Kp30, Kp55, Kp140, Kp290, Kp296).

5.4 Discussion

K. pneumoniae has become one of the most important causes of nosocomial and community acquired infections. Aminoglycosides, β-lactams mainly extended spectrum cephalosporins and carbapenems (amikacin, cefepime, and carbapenem) constitute the main therapeutic choices to treat infections caused by these pathogens in NICU, JNM College and hospital, Aligarh. However, resistance to these compounds has been increasingly reported from different parts of the world in recent years [Cantón *et al.*, 2008]. Among the 262 *K. pneumoniae* strains, 15 were multidrug resistant (MDR) (i.e. resistant to three classes of antimicrobial agents like β-lactam, aminoglycosides, fluoroquinolones and cepalosporins. In one of this studies carried in Malaysia, the rate of MDR *K. pneumoniae* strains was found to be 53% which was consistent to our study [Manchanda *et al.*, 2005].

The present data shows the resistance against third generation cephalosporins among *K. pneumoniae* isolates. 86.64% isolates of *K. pneumoniae* were found to be resistant to cefazolin, 79.00% to cefuroxime, 67.93% to cephotaxime and 65.26% resistant to ceftriaxone. It could be due to some other resistance mechanism such as lack of permeation of porins and AmpC β lactamase production. Our data are comparable to earlier studies [Mohamudha *et al.*, 2010]. The higher proportion of resistant to tetracycline (72.51%) and cotrimoxazole (85.11%) is mainly due to widely used antibiotics over the past decade in this region. Similar studies have also been reported in other parts of the country. Our data have also shared harmony to previous reports [Shobha *et al.*, 2007].

Our study revealed the high prevalence of increasing resistance to inhibitors like amoxyclav and antibiotics in combination with inhibitors among *K. pneumoniae* isolates. 92.74% isolates of *K. pneumoniae* were found to be resistant to amoxyclav, 86.78% to ampicillin/sulbactum, 72.13% to amoxicillin/sulbactum, 59.92% to cefixime/clavulanic acid and 49.61% to cefepime/tazobactam. This is consistent with previous finding that *K. pneumoniae*, overproduction of either TEM or SHV increased resistance to amoxicillin-clavulanic acid and to cephalothin [Petit *et al.*, 1992]. Findings of present study revealed that, among the 15 ESBL producing *K. pneumoniae* strains, all are positive for $\boldsymbol{bla}_{\text{CTX-M}}$

and bla_{OXA-1} whereas, 10 for bla_{SHV} and 13 for bla_{TEM}. The presence of multiple ESBL encoding genes in *K. pneumoniae* has also been reported by Romero *et al* in 2007. His study had also reported the co-existence of $bla_{CTX-M-15}$ and bla_{OXA-1} [Romero *et al.*, 2007]. Our data demonstrate that ESBL producing *K. pneumoniae* isolates from NICU showed bla_{CTX-M}, bla_{TEM}, bla_{SHV} and bla_{OXA-1} association in the same strain (Table 3). The $bla_{CTX-M-15}$ and bla_{OXA-1} association in the same strain has also been reported in USA [Hradecka *et al.*, 2008].

In the present study, the percent resistance against aminoglycoside was 55.85% while 43.12% isolates shared resistance against amikacin. These amikacin resistant isolates were found to possess 16s RNA methylase gene and 53.33% of these isolates exhibited high level of amikacin, whereas, 40% of isolates represent amikacin, ciprofloxacin, gatifloxacin, cephotaxime, ceftazidime and cefepime MDR phenotype indicating significant effect of 16s RNA methylases on the emergence of amikacin resistant strains among the ESBL producing *K. pneumoniae* isolates. Our studies are comparable to the study conducted by Corvec *et al* (2009).

The resistance to β- lactamase inhibitors has been improved by the combined production of CTX-M and OXA enzymes in *E. coli* and *K. pneumoniae.* This presumably explains their non-susceptibility to amoxicillin/clavulanate [Livermore *et al.*, 2005] ESBLs producers is becoming a serious clinical problem for the clinician and physician to treat infection. In this study, aminoglycosides, fluoroquinolone, β lactam, cephalosporin and monobactum resistant genes were found to transfer through horizontal gene transfer in all the tested clinical isolates. CTX-M producing isolates were found resistant to cefepime (MIC value ranging 7.81µg/ml). The cefepime is hydrolyzed by CTX-M with higher efficiency than other ESBL types [Mshana *et al.*, 2009].

Plasmid analysis revealed that majority of the transconjugants harbored large plasmids of 154kb, 64kb and 28kb. In earlier studies bla_{CTX-M} gene was reported to be present on the varying range of plasmid (7 to 200 kb) [Baudry *et al.*, 2009]. Our study also revealed varying sized plasmids carrying bla_{CTX-M} and other resistant markers which are co-transferred during conjugation among the *K. pneumoniae* isolates.

ERIC typed strains (Ab, Ac, Ad, Ae, Af, Ah, Ak, Al, Am, An and Ao) were subjected to undergo replicon typing. Five (Inc/I1γ, Inc/FIA-FIB, Inc/FIB, Inc/HI2 and Inc/HI1) different replicon types were identified, carrying $bla_{CTX\text{-}M\text{-}3}$ $bla_{TEM\text{-}1}$, $bla_{SHV\text{-}1}$ and $bla_{OXA\text{-}1}$ genes which have already been shown in earlier studies [Pe´rez *et al.*, 2009; Poirel *et al.*, 2007; Novais *et al.*, 2006 & Hradecka *et al.*, 2008].

5.5 Conclusions

This study concludes polyclonal dissemination of ESBL producing strains among clinical isolates of *K. pneumoniae* in a teaching hospital of north India. Moreover, this study also suggests carbapenem, ceftazidime and amikacin as the drugs of choice for the present community. Furthermore, efforts are needed to promote the proper use of antibiotics and to discourage their sale over the counter.

Chapter-6

Screening of MBL producers in diabetic foot ulcer patients

6.1 Introduction

Diabetic foot ulcer is the most alarming health problem in India and is expected to increase to million populations by 2025 [Abdul *et al.*, 1999]. It has also been reported that 15% of diabetic patients would develop a foot ulcer during the course of their disease [Boulton *et al.*, 2004]. Neuropathy and impaired blood supply in combination with deformities of the feet and the resulting increased pressures on areas of the sole are the most important pathophysiological factors. Though, diabetic foot infection is a broader term that includes many pathologies but the most common form of diabetic foot infection is diabetic foot ulcer which mainly results from neuropathy, ischemia and other complex mechanism [Lipsky *et al.*, 2004]. In diabetic foot ulcer protective layer of skin is breached and the underlying soft tissues were exposed, thus, providing a better media for bacterial colonization [Schubert & Heesemann., 1995]. Infection of diabetic foot ulcer by *Staphylococcus* has been predominantly observed [Xu *et al.*, 2007; Yates *et al.*, 2009]. *Pseudomonas aeruginosa* [Martínez-Gómez *et al.*, 2009], *E. coli* [Varaiya *et al.*, 2008], *Klebsiella* species [Goldstein *et al.*, 2008], *Enterococcus* species [Martínez-Gómez *et al.*, 2009], and *Proteus* species [Raja *et al.*, 2007] have also been reported in various diabetic foot ulcer cases.

The recent emergence of antibiotic resistance in bacterial pathogens, both nosocomially and in community is very serious development that threatens the end of the antibiotic era [Rodriguez-Bano & Navarro., 2008; Shakil *et al.*, 2008; Peterson & Bonomo., 2005] .More than 70% of the bacteria associated with hospital acquired infections in United States are resistant to one or more of the drugs previously used to treat them [Levy & Marshall., 2004]. The rise in multi drug resistance organism attracted the world wide biomedical researchers as the available treatment has higher degree of failure and high morbidity associated with the infection [Gupta & Stamm., 2002; Sobel & Kaye., 2004. Also, several studies support the prevalence of extended spectrum β-lactamase (ESBL) producing multidrug resistant gram negative bacteria in diabetic foot ulcer. Appearance of multiple β-lactamases in same clinical isolates becomes a challenge for clinicians and researchers as it is more difficult to detect the causative resistance factors in multidrug resistance pathogens [Karen Bush & Jed F. Fisher., 2011]. However, β-Lactams are still

the drug of choice for serious infective condition and the most active of these are the carbapenems, which are specially prescribed in ESBL producing pathogen mediated infection like *Escherichia coli* and *Klebsiella pneumoniae* [Young *et al.*, 2009].

The metallo-β-lactamases (zinc dependant carbapenemases) are bacterial enzymes which hydrolyze the carbapenems owing to resistance to the β-lactamase inhibitors but become inhibited by metal ion chelators like EDTA. Most of these enzymes hydrolyze the cephalosporins and penicillins. Monobactam was found to be susceptible in MBL producing *K. pneumoniae* isolates. The most common metallo-β-lactamase families include the VIM, IMP, GIM, and SIM enzymes that are located within a variety of integron structures, where they have been incorporated as gene cassettes. When these integrons become associated with plasmids or transposons, transfer between bacteria is readily facilitated. A new subgroup of metallo-β-lactamase (MBL), designated as New Delhi metallo-β-lactamase (NDM-1), originating from New Delhi, India which was first reported from a Swedish patient of Indian origin who travelled to New Delhi, India, and acquired a urinary tract infection caused by a carbapenem resistant *K. pneumoniae* strain [Yong *et al.*, 2009]. There are several reports about the occurrence of NDM-1 MBL producing bacterial isolates in many parts of the world but mostly they represent the bacterial population of Indian origin [Bratu *et al.*, 2005; Da Silva *et al.*, 2002; Danel *et al.*, 1997; Ambler *et al.*, 1991; Gibb *et al.*, 2002; Naas *et al*, 1994; Bratu *et al.*, 2005]. Various works has been carried out to explore and to characterize the MBL producing bacterial isolates but there is no study regarding acquisition of MBL producing isolates in diabetic foot ulcerated patients. Therefore, this study was carried out to identify the multiple β-lacatmase specially, MBL producing bacterial isolates in diabetic foot ulcerated patient admitted to the endocrinology ward of Aligarh hospital, a tertiary care hospital of north India. The study aimed to determine the resistance and susceptibility of those isolates, and to characterize the mode of transmission of bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{NDM-1}, bla_{OXA} and ArmA, among the MBL producing clinical isolates from infected diabetic foot ulcers.

6.2 Experimental outline

Twenty diabetic patients having clinically infected foot ulcers admitted to the endocrinology ward and ICU at the J. N. Medical College and hospital, Aligarh, over a period of 1 year (April 2010 to November 2011) were studied in a consecutive series. Wagner classification was employed to grade the ulcers [Wagner, 1981]. If a patient had multiple ulcers, specimens from all the ulcers were taken; and the patient was grouped in the multidrug resistance gram negative bacteria (MDRGNB) infected category if any of the ulcers was found to be MDRGNB positive. Informed consent was obtained from all subjects, and clearance was obtained from the institute's ethics committee. Culture specimens were obtained at the time of admission, after the surface of the wound had been washed vigorously by saline, and followed by debridement of superficial exudates. Specimens were obtained by scraping the ulcer base or the deep portion of the wound edge with a sterile curette. The soft tissue specimens were quickly sent to the laboratory and processed for microbial pathogens, which were identified as outlined in section 2.2.1. Final confirmation of bacterial identities was done by 16S rDNA sequencing as mentioned in section 2.2.1. Antimicrobial susceptibility testing of the isolates was performed by the CLSI standard disc diffusion method as outlined in section 2.2.2. The ESBL and MBL phenotypic confirmatory test was performed as mentioned in section 2.2.2. MICs of Monobactam, cephalothin, cefazolin, cefuroxime, cephoxitine, ceftazidime, cephotaxime, cefepime, imipenem, meropenem, ertapenem, dorapenem, colistin, tigecyclin and aztreonam were determined by the CLSI microbroth dilution method. A search for bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{OXA-1}, bla_{NDM-1} and Arm-A genes was performed by PCR amplification. The presence of insertion sequences (IS) known to be associated with bla_{NDM-1} in *Enterobacterial* isolates from endocrinology ward of Aligarh hospital, were confirmed by using oligonucleotide specific primers as listed in table 2.1 for both genomic and plasmid DNA. PCR-based replicon typing was carried out on 2 non-conjugative plasmids as described in chapter 4. MLST was performed according to the protocol described in section 2.2.12. Transmissibility of bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{OXA-1}, bla_{NDM-1} and Arm-A markers were checked by conjugation as described in section 2.2.4. Sequencing was done as mentioned in section 2.2.10. Gen Bank accession

numbers of the KP 12 and EC 15 genes sequenced in this study are JN680194.1 and JN860195.1, respectively.

6.3 Results

Twenty diabetic patients having clinically infected foot ulcers admitted to the endocrinology ward at the J. N. Medical College and hospital, Aligarh, over a period of 1 year (April 2009 to November 2010) were studied. Among those, 14 (70%) were *K. penumoniae*, 5 (25%) were *E. coli* and one (5%) was *E. cloacae*. Of 14 *K. penumoniae*, one clinical isolates was carbapenem (imipenem, meropenem, ertapenem and dorapenem) resistant whereas the single *E. cloacae* strain was also carabapenem resistant. All clinical isolates were found to be resistant to β-lactam groups (100%), aminoglycoside (90%), fluoroquinolones (75%), cephalosporin (71%), other β-lacam antibiotics (81%) and carbapenem group (21%). Monobactam group were found to be susceptible against these isolates (100%).

Minimum inhibitory concentrations of β-lacatm, aminoglycoside, 1st, 2nd, 3rd and 4th generation of cephalosporins and carbapenem were observed to be 250μg/ml, 7.81-250 μg/ml, 15.625-250μg/ml and 1.95-31.25μg/ml, respectively (Table 6.2). Colistin and tigecycline were found to be susceptible to these isolates (Table 6.2).

PCR amplification and sequence analysis of DNA from these clinical isolates, using primers as described previously (Table 2.1), revealed the presence of bla_{CTX-15}, (Figure 6.1) bla_{SHV-1} (Figure 6.3) in all clinical isolates, bla_{TEM-1} (Figure 6.2) in 12 isolates (9 isolates or 75% in *K. pneumoniae*, 2 isolates or 16.7% in *E. coli* and one isolate or 8.3% in *E. clocace*) and bla_{OXA-1} (Figure 6.4) in 7 isolates (5 isolates or 71.4% in *K. pneumoniae*, one isolate or 14.3% in *E. coli* and one isolate or 14.3% in *E. clocace*). bla_{NDM-1} (Figure 6.6) was identified in Ec15 and Kp12 strain only. In addition, a 16s rRNA methylase gene (Arm-A) was also detected in 5 clinical isolates (Figure 6.5) (4 isolates or 80% in *K. pneumoniae* and one isolate or 20% in *E. clocace*). No bla_{OXA-48}, bla_{IMP}, bla_{VIM} or bla_{KPC} genes were amplified from these isolates. After the PCR amplification, Kp12 and Ec15 were identified as bla_{NDM-1} producer isolates and subjected for further study. Conjugal transfers were performed using Kp12 and Ec15 isolates

(Figure 6.7). Three types of transconjugants were obtained for Kp12 and two types were obtained for Ec15, according to their resistance phenotypes. One of the transconjugants of Kp12 showed ESBL and NDM-1 producing phenotypes, second transconjugant showed only an ESBL producing phenotype and the third showed only the NDM-1 producing phenotype. Ec15 transconjugants showed two types of phenotypes, one displaying an ESBL and NDM-1 producing phenotype and the other displaying a NDM-1 producing phenotype. Tests using a PCR based replicon typing method (PBRT) revealed that the bla_{NDM-1} carrying plasmid in Ec15 was of incompatibility group Inc L/M (Figure 6.9, Table 6.3), whereas the plasmid in Kp12 carrying the bla_{NDM-1} determinant was found untypeable. Multilocus sequence typing (MLST) was performed for Kp12 (Figure 6.8), as described earlier which was found to be of the type ST14. Primers targeting the IS element A*ba*125 identified a remnant of ISA*ba*125 upstream of the bla_{NDM-1} gene in the Ec15, whereas an entire ISA*ba*125 element was identified upstream of the bla_{NDM-1} gene in the Kp12 (Figure 6.10). A bleomycin resistance gene *ble*MBL was identified downstream of the bla_{NDM-1} gene in both cases (Figure 6.11).

Table 6.1 Susceptibility pattern of antibiotics among different clinical isolates of diabetic foot ulcer

Antibiotic groups		Anti-biotics	Susceptibility pattern																			% resistance to antibiotics
			Kp1	Kp2	Kp3	Kp4	Ep5	Kp6	Kp7	Kp8	Ep9	Kp10	Ep11	Kpl2	Kp13	Kp14	Ec15	Kp16	Kp17	Ep18	Kp19	Ep20	
Aminoglycosides		G	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	20(100.00)
		Tb	R	R	R	R	R	S	R	R	R	S	R	R	R	R	R	R	R	R	R	R	18(90.00)
		Ak	R	R	R	S	R	R	S	R	R	R	R	R	S	R	R	R	R	S	R	R	16(80.00)
Fluoroquinolones		Na	R	R	R	R	R	S	R	R	R	R	R	S	R	R	S	R	R	R	S	S	16(80.00)
		Cf	R	S	R	R	R	S	R	R	R	S	R	R	R	R	R	S	R	S	R	S	14(70.00)
		Gf	R	R	S	R	R	S	R	S	R	R	S	R	R	S	R	R	R	R	R	R	15(75.00)
β-Lactams		A	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	20(100.00)
		P	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	20(100.00)
		Pc	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	20(100.00)
Others		T	R	R	S	R	R	R	S	R	S	R	R	R	R	S	R	R	S	R	R	R	15(75.00)
		Co	R	S	R	R	S	R	R	S	R	S	R	R	R	S	R	S	R	R	R	R	14(70.00)
		Ox	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	20(100.00)
		Ch	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	19(95.00)
Cephalosporins	1st g	Cz	R	R	S	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	18(90.00)
		Cu	R	R	S	R	S	R	S	R	R	R	R	R	R	S	R	R	R	R	R	R	16(80.00)
	2nd g	Cn	S	R	S	R	S	R	S	R	R	R	R	R	R	R	S	R	R	R	R	R	15(75.00)
		Ca	R	R	S	R	S	S	R	R	R	R	R	R	S	R	S	R	R	S	R	R	14(70.00)
	3rd g	Ce	S	R	S	S	R	R	R	R	R	S	R	R	S	R	R	R	S	S	R	R	13(65.00)
		Cl	S	R	S	S	R	R	R	S	R	R	S	R	R	R	R	R	S	S	R	S	12(60.00)
	4th g	Cpm	S	R	S	S	R	R	S	R	S	R	S	R	S	R	R	R	S	S	R	S	10(50.00)
Carbapenems		Imp	S	R	S	S	R	S	S	S	S	S	S	R	S	S	R	S	S	S	S	S	4(02.00)
		Mr	S	S	S	R	S	S	R	S	S	S	S	R	S	R	R	S	R	S	S	S	6(03.00)
		Ert	S	S	S	S	S	S	S	R	S	S	S	R	R	S	R	S	S	S	S	R	5(02.50)
		Dor	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S	S	S	S	S	2(01.00)
Monobactam		Col	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	20(100.00)
		Tig	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	20(100.00)
		Ao	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	20(100.00)

Generation (g),Gentamicin (G), Tobramycin (Tb), Amikacin (Ak), Nalidixicacid (Na), Generation (g), Ciprofloxacin (Cf), Gatifloxacin (Gf), Ampiciln (A), Penicillin (P), Pipracillin (Pc),Tetracyclin (T), Cotrimoxazole (Co), Oxacillin (Ox), Cephalothin (Ch),Cefazolin (Cz), Cefuroxime (Cu), Cephoxitine (Cn), Ceftazidime (Ca), Cephotaxime (Ce), Ceftriaxone (Ci), Cefepime (Cpm), Imipenem (Imp), Meropenem (Mr), Ertapenem (Ert), Dorapenem (Dor), Colistin (Col), Tigecyclin (Tig), Aztreonam (Ao), Resistance (R) , Susceptible (S).

Table 6.2 MICs of different clinical isolates of diabetic foot ulcer

S.no	MIC(µg/ml)																	
	β-lactams			Aminoglycosides			Cephalosporins								Carbapenems			
	A	P	Pc	G	Tb	Ak	Ch	Cz	Cu	Cn	Ca	Ce	Cl	Cpm	Imp	Mr	Ert	Dor
Kp1	250	250	250	15.625	62.5	250	250	250	125	125	62.5	62.5	31.25	31.25	1.95	1.95	1.95	1.95
Kp2	250	250	250	7.81	31.25	250	15.625	31.25	15.626	15.625	31.25	15.625	3.90	1.95	1.95	1.95	1.95	1.95
Kp3	250	250	250	15.625	62.5	250	31.25	15.626	15.626	15.625	31.25	15.625	3.90	1.95	1.95	1.95	1.95	1.95
Kp4	250	250	250	125	62.5	62.5	31.25	31.25	15.625	15.625	31.25	15.625	3.90	1.95	1.95	1.95	1.95	1.95
Ep5	250	250	250	15.625	62.5	250	250	250	125	125	62.5	62.5	31.25	31.25	1.95	1.95	1.95	1.95
Kp6	250	250	250	250	250	250	250	125	62.5	62.5	31.25	31.25	15.625	15.625	1.95	1.95	1.95	1.95
Kp7	250	250	250	7.81	31.25	250	15.625	31.25	15.626	15.625	31.25	15.625	3.90	1.95	1.95	1.95	1.95	1.95
Kp8	250	250	250	15.625	62.5	250	250	250	125	62.5	62.5	31.25	31.25	15.625	1.95	1.95	1.95	1.95
Ep9	250	250	250	250	250	125	125	62.5	62.5	31.25	15.626	15.625	31.25	15.625	1.95	1.95	1.95	1.95
Kp10	250	250	250	250	125	62.5	62.5	31.25	31.25	15.626	15.625	15.625	7.81	7.81	1.95	1.95	1.95	1.95
Ep11	250	250	250	250	250	250	250	125	62.5	62.5	31.25	31.25	15.625	15.625	1.95	1.95	1.95	1.95
Kpl2	250	250	250	15.625	62.5	250	250	250	125	125	62.5	62.5	31.25	31.25	3.90	3.90	31.25	3.90
Kp13	250	250	250	7.81	31.25	250	15.625	31.25	15.626	15.625	31.25	15.625	3.90	1.95	1.95	1.95	1.95	1.95
Kp14	250	250	250	250	250	250	250	125	62.5	62.5	31.25	31.25	15.625	15.625	1.95	1.95	1.95	1.95
Ec15	250	250	250	15.625	250	250	250	250	125	62.5	62.5	31.25	31.25	15.625	3.90	31.25	15.625	15.625
Kp16	250	250	250	250	7.81	31.25	31.25	31.25	31.25	31.25	31.25	31.25	15.625	15.625	1.95	1.95	1.95	1.95
Kp17	250	250	250	7.81	31.25	250	15.625	31.25	15.626	15.625	31.25	15.625	3.90	1.95	1.95	1.95	1.95	1.95
Ep18	250	250	250	15.625	62.5	250	31.25	15.626	15.625	31.25	15.625	3.90	1.95	1.95	1.95	1.95	1.95	1.95
Kp19	250	250	250	250	125	125	62.5	62.5	31.25	31.25	15.625	15.625	7.81	7.81	1.95	1.95	1.95	1.95
Ep20	250	250	250	15.625	62.5	250	250	250	125	62.5	62.5	31.25	31.25	15.625	1.95	1.95	1.95	1.95

Ampicillin (A), Penicillin (P), Pipracillin (Pc), Gentamicin (G), Tobramycin (Tb), Amikacin (Ak), Cephalothin (Ch), Cefazolin (Cz), Cefuroxime (Cu), Cephoxitine (Cn), Ceftazidime (Ca), Cephotaxime (Ce), Ceftriaxone(Ci), Cefepime (Cpm), Imipenem (Imp), Meropenem (Mr), Ertapenem (Ert), Dorapenem (Dor).

Table 6.3 Genetic analysis of NDM-1 and other resistant marker on plasmids

S.no	Resistance marker	ISAba125	*ble* MBL	ST-Type	Plasmid carrying bla_{NDM-1}
Kp1	bla_{CTX-15}, bla_{SHV-1}, bla_{TEM-1}				
Kp2	bla_{CTX-15}, bla_{SHV-1}, bla_{OXA-1}				
Kp3	bla_{CTX-15}, bla_{SHV-1}, bla_{TEM-1}				
Kp4	bla_{CTX-15}, bla_{SHV-1}, bla_{TEM-1},Arm-A				
Ep5	bla_{CTX-15}, bla_{SHV-1}				
Kp6	bla_{CTX-15}, bla_{SHV-1}, bla_{TEM-1}				
Kp7	bla_{CTX-15}, bla_{SHV-1}, bla_{OXA-1}				
Kp8	bla_{CTX-15}, bla_{SHV-1}, bla_{OXA-1}				
Ep9	bla_{CTX-15}, bla_{SHV-1}				
Kp10	bla_{CTX-15}, bla_{SHV-1}				
Ep11	bla_{CTX-15}, bla_{SHV-1}, bla_{TEM-1}				
Kpl2	bla_{CTX-15}, bla_{SHV-1}, bla_{TEM-1} bla_{OXA-1}, bla_{NDM-1},Arm-A	+	+	ST-14	Unable to type by PBRT method
Kp13	bla_{CTX-15}, bla_{SHV-1}, bla_{OXA-1} ,Arm-A				
Kp14	bla_{CTX-15}, bla_{SHV-1}, bla_{TEM-1}				
Ec15	bla_{CTX-15}, bla_{SHV-1}, bla_{TEM-1} bla_{OXA-1}, bla_{NDM-1}, Arm-A	Truncated	+	-	Inc L/M
Kp16	bla_{CTX-15}, bla_{SHV-1}, bla_{TEM-1}				
Kp17	bla_{CTX-15}, bla_{SHV-1}, bla_{TEM-1}				
Ep18	bla_{CTX-15}, bla_{SHV-1}, bla_{OXA-1}				
Kp19	bla_{CTX-15}, bla_{SHV-1}, bla_{TEM-1}, Arm-A				
Ep20	bla_{CTX-15}, bla_{SHV-1}, bla_{TEM-1}				

+, Positive; –, not determined; ST, sequence type; Inc, incompatibility.

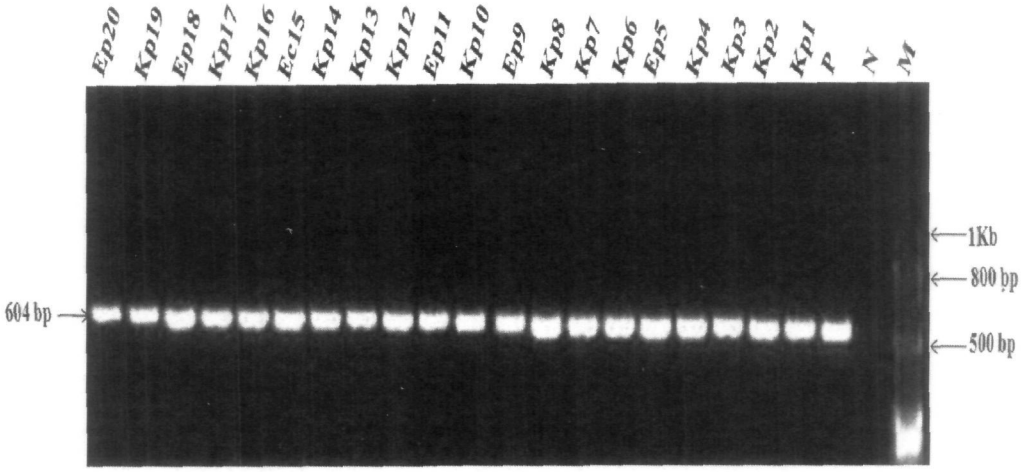

Figure 6.1 PCR amplification of *bla*$_{CTX-M}$ gene, Lane 1-20 clinical isolates of diabetic foot ulcer (Ep20, Kp19, Ep18, Kp17, Kp16, Ec15, Kp14, Kp13, Kp12, Ep11, Kp10, Ep9, Kp8, Kp7, Kp6, Ep5, Kp4, Kp3, Kp2, Kp1), Lane P: positive control Lane N: negative control and Lane M: 1 Kb DNA ladder,

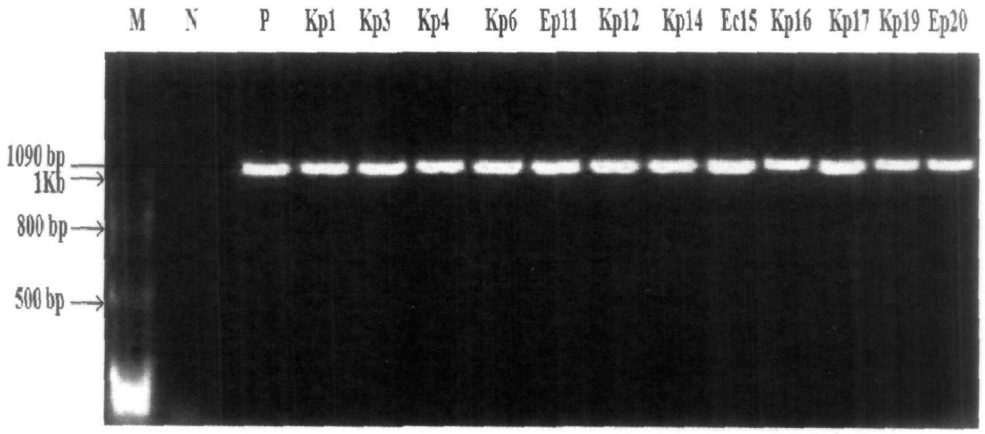

Figure 6.2 PCR amplification of *bla*$_{TEM}$ gene, Lane M: 1 Kb DNA ladder, Lane N negative control, Lane P: positive control and 1-12 clinical isolates of diabetic foo ulcer (Kp1, Kp3, Kp4, Kp6, Ep11, Kp12, Kp14, Ec15, Kp16, Kp17, Kp19, Ep20).

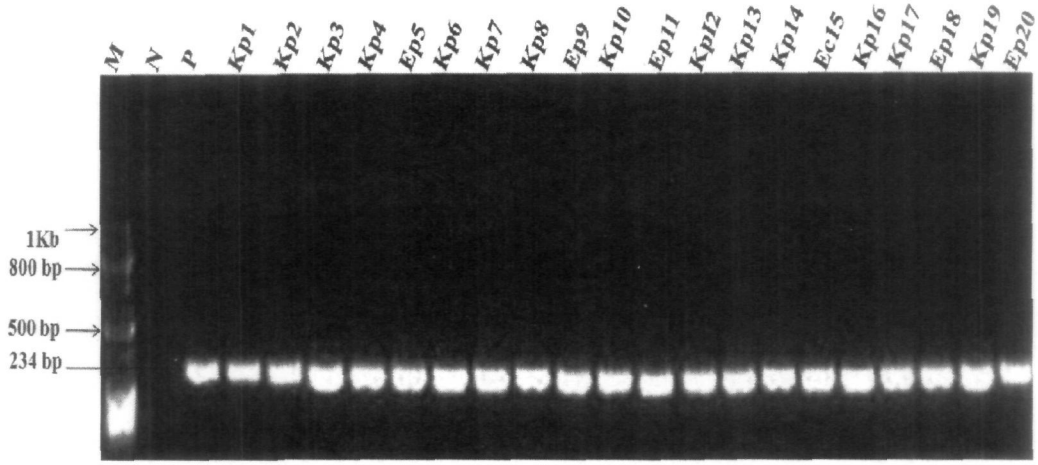

Figure 6.3 PCR amplification of *bla*$_{SHV}$ gene, Lane M: 1 Kb DNA ladder, Lane N: negative control, Lane P: positive control and 1-20 clinical isolates of diabetic foot ulcer (Kp1, Kp2, Kp3, Kp4, Ep5, Kp6, Kp7, Kp8, Ep9, Kp10, Ep11, Kp12, Kp13, Kp14, Ec15,Kp16, Kp17,Ep18, Kp19, Ep20).

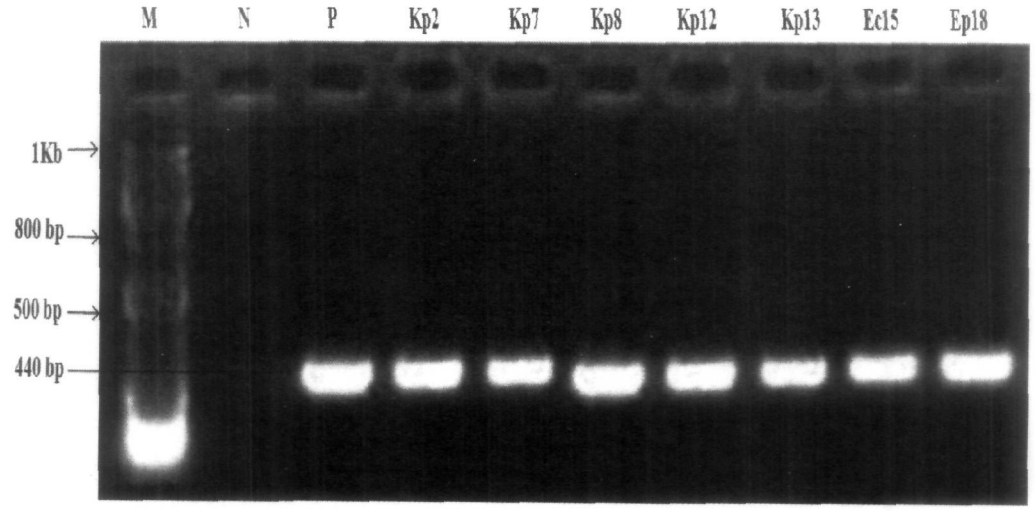

Figure 6.4 PCR amplification of *bla*$_{OXA-1}$ gene, Lane M: 1 Kb DNA ladder, Lane N: negative control, Lane P: positive control and 1-7 clinical isolates of diabetic foot ulcer (Kp2, Kp7, Kp8, Kp12, Kp13, Ec15, Ep18).

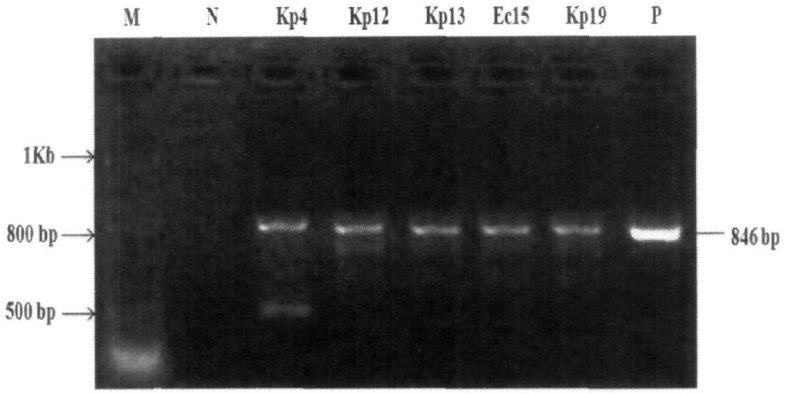

Figure 6.5 PCR amplification of Arm-A gene, Lane M: 1 Kb DNA ladder, Lane N: negative control, lane 1-5 clinical isolates of diabetic foot ulcer (Kp4, Kp12, Kp13, Ec15, Kp19) and lane P: positive control.

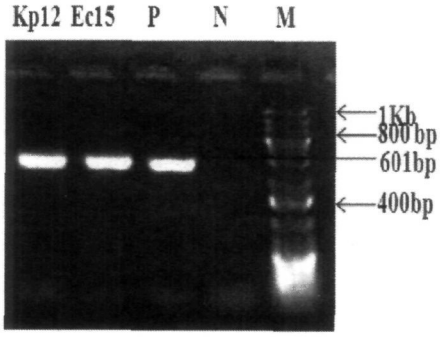

Figure 6.6 PCR amplification of NDM-1 gene, lane1-2 clinical isolates of diabetic foot ulcer (Kp12, Ec15), lane P: positive control and lane N: negative control.

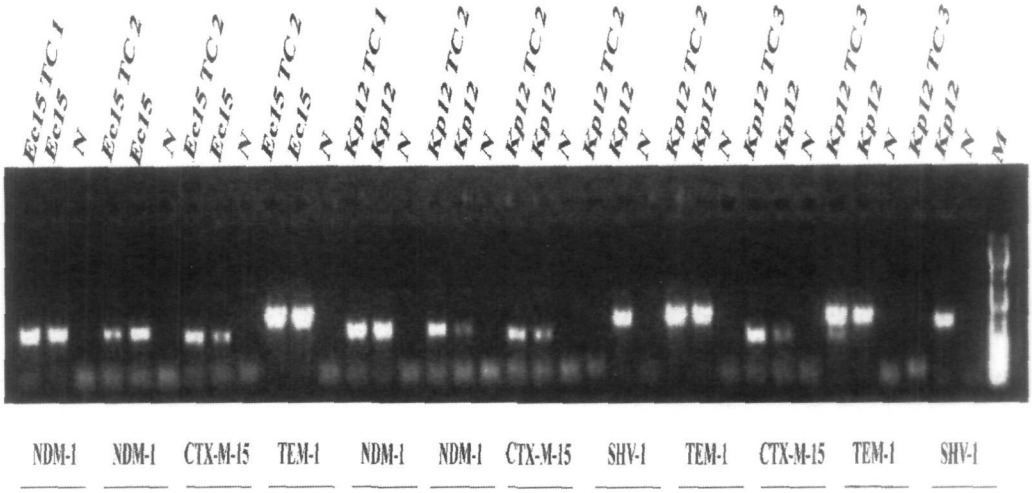

Figure 6.7 PCR amplification of resistant markers obtained from Kp12 and Ec15 to confirm the transfer of markers by transconjugation.

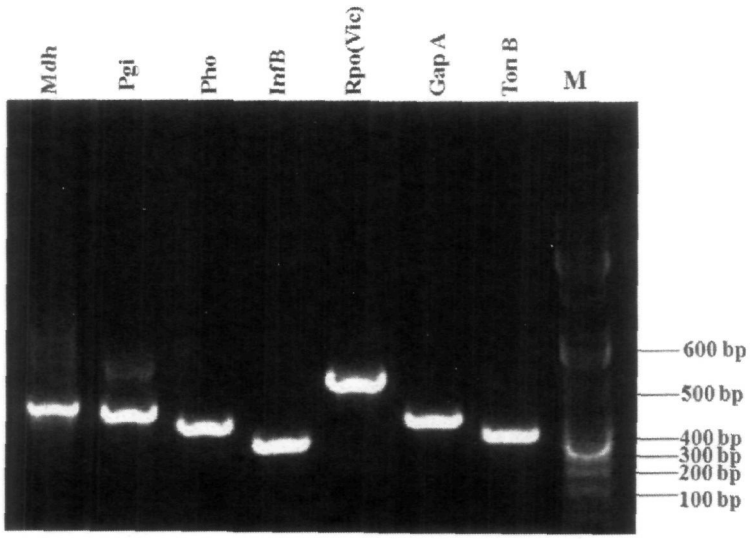

Figure 6.8 PCR amplification of MLST profile of KP12 strain from clinical isolate (diabetic foot ulcer). The gel shows lane *mdh*, *pgi*, *pho*, *inf*B, *rpo*, *gap*A, *ton*B with their corresponding molecular sizes 477bp, 432bp, 420pb, 318bp, 501bp, 450bp and 414bp respectively.

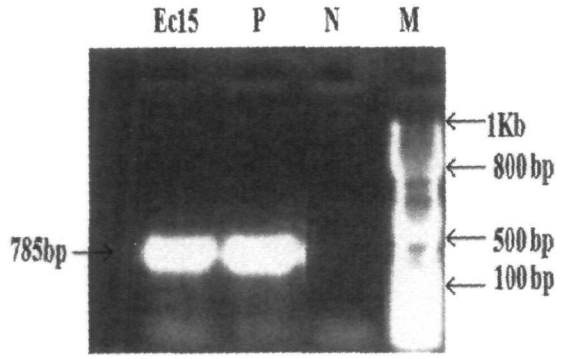

Figure 6.9 PCR amplification of Incompatibility (L/M) profile from clinical isolate (diabetic foot ulcer). Lane 1 (Ec15 clinical isolates), lane P: positive control and lane M: 1Kb marker.

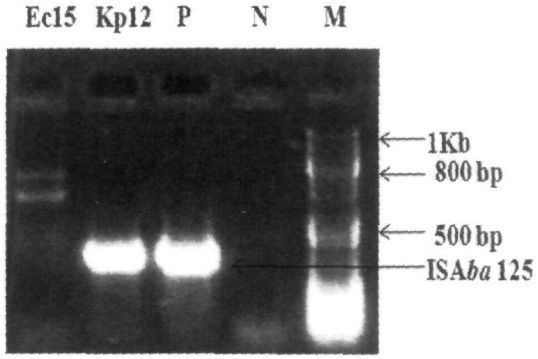

Figure 6.10 PCR amplification of ISA*ba*125 profile from clinical isolate (diabetic foot ulcer). Lane 1-2 (Ec15, Kp12 clinical isolates), lane P: positive control and lane M: 1Kb marker.

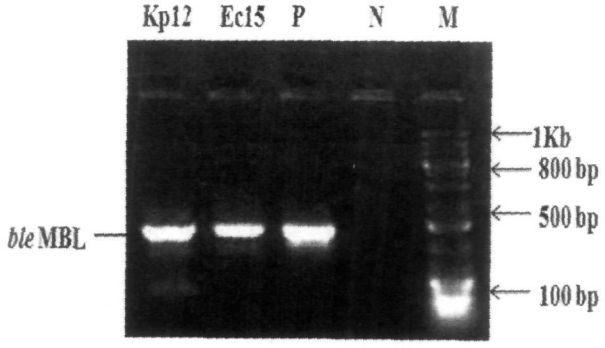

Figure 6.11 PCR amplification of *ble*MBL profile from clinical isolate (diabetic foot ulcer). Lane 1-2 (Ec15, Kp12 clinical isolates), lane P: positive control and lane M: 1Kb marker.

6.4 Discussion

Infective diabetic foot ulcer is the most common single precursor to amputation and has been identified as a component in 85% of lower extremity amputations [Margolis *et al.*, 2005]. Over 20-40% diabetic wounds have been found to be MRSA infected by many studies [Shanker *et al.*, 2005; Tentolouris *et al.*, 2006]. Multi drug resistance becomes a worldwide problem for clinicians as well as patients. Appearance of multiple β-lactamases in same clinical isolates also becomes a more worrisome to clinicians and researchers [Karen Bush & Jed F. Fisher., 2011]. Carbapenems are the last resort drugs for ESBL producing pathogen mediated infection like *Escherichia coli* and *Klebsiella pneumoniae* [Young *et al.*, 2009].

The present study which was conducted on infective diabetic foot ulcer patient admitted in ICU and endocrinology ward of JNMCH, Aligarh revealed that out of twenty clinical isolates, 14 were *K. pneumoniae,* 5 were *E. coli* and one was *E. cloacae*. Our study demonstrates the higher prevalence of *K. pneumoniae* in diabetic foot ulcers as compared to *E. coli* and *E. Cloacae* at JNMCH, Aligarh.

The identified strains were assessed for resistance behaviour against therapeutically used antibiotics. Of 14 *K. penumoniae,* one cilinical isolate (Kp12) was carbapenem resistant whereas single *E. cloacae* (Ec15) strain was also carbapenem (imipenem, meropenem, ertapenem and dorapenem) resistant. All clinical isolates, including Kp12 and Ec15, were found to be resistant to β-lactam (100%), aminoglycoside (90%), fluoroquinolones (75%), cephalosporin (71%), other β-lacam antibiotics (81%) and carbapenem group (21%). Monobactam group was found to be susceptible against these isolates (100%). Our data are comparable with the earlier studies of Mohamudha *et al.*, (2010), Shobha *et al.*, (2007) and Sader *et al.*, (1998).

The present data shows that all clinical isolates were resistant against multiple groups of antibiotics (β-lactam, aminoglycosides, fluoroquinolone and cephalosporin). This is consistent with previous findings [Bizzarro *et al.*, 2007; Manchanda *et al*, 2005].

In the present study, clinical isolates were found to be highly resistant to β-lactam groups (100%) and aminoglycoside (90%). Resistance of these antibiotics may be justified on the basis of cost effect and freely accessibility in local market of this region. Similar

studies have also been carried out in other parts of India indicating the shared harmony to our study [Shobha *et al.*, 2007].

Minimum inhibitory concentrations of β-lacatm, aminoglycoside, 1st, 2nd, 3rd and 4th generation of cephalosporin and carbapenem in these clinical isolates were observed to 250μg/ml, 7.81-250 μg/ml, 15.625-250μg/ml and 1.95-31.25 μg/ml, respectively (Table 6.2). Colistin and tigecycline were found to be susceptible to these isolates (Table 6.2). MICs behaviour of these clinical isolates substantiates our findings in above mentioned drug resistance study.

There are several studies showing the reason and mechanism of resistance of ESBL producers isolates to these conventional antibiotics. But resistance of two isolates Kp12 and Ec15 to carbapenem group of antibiotics directed our study to explore the possible character and mechanism of all clinical isolates. So, metallo-β-lactamase activity was carried out in all clinical isolates. And after confirmation of MBL producing activity, PCR amplification and sequence analysis of DNA revealed the presence of bla_{CTX-15}, bla_{SHV-1} in all clinical isolates, bla_{TEM-1} in 12 isolates (9 isolates or 75% in *K. pneumoniae*, 2 isolates or 16.7% in *E. coli* and one isolate or 8.3% in *E. clocace*) and bla_{OXA-1} in 7 isolates (5 isolates or 71.4% in *K. pneumoniae*, one isolate or 14.3% in *E. coli* and one isolate or 14.3% in *E. clocace*). In addition, a 16s rRNA methylase gene (ArmA) was also detected in 5 clinical isolates (4 isolates or 80% in *K. pneumoniae* and one isolate or 20% in *E. clocace*). No bla_{OXA-48}, bla_{IMP}, bla_{VIM} or bla_{KPC} genes were amplified in these isolates, whereas they have been reported previously in *Enterobacterial* isolates from India [Poirel *et al.*, 2011b]. Major finding of PCR amplification was the presence of bla_{NDM-1} in Kp12 and in Ec15, therefore, these two isolates subjected for further study.

In conjugal transfer study, three types of transconjugants were obtained for Kp12 and two types for Ec15, according to their resistance phenotypes. One of the transconjugants of Kp12 strain showed ESBL and NDM-1 producing phenotypes, other showed only an ESBL producing phenotype and the third showed only the NDM-1 producing phenotype. Two types of transconjugants, one displaying an ESBL and NDM-1 producing phenotype and the other displaying a NDM-1-producing phenotype, were obtained for Ec15 strain. These findings may explore the possible mechanism of carbapenem producers.

Plasmid profiling, revealed that Kp12 harboured three plasmids whereas Ec15 carried two plasmids, all ranging from 66 to 154 kb in size. Varying sized plasmid (5-185 kb) carrying ESBL genes have also been detected in *K. pneumoniae* in earlier studies [Essack *et al.*, 2001].

PCR-based replicon typing (PBRT) revealed that the NDM-1carrying plasmid in Ec15 was of incompatibility group Inc L/M (Table 6.3), whereas the plasmid in Kp12 carrying the NDM-1 determinant was not able to be typed. The study of these isolates showed that the gene coding for the widespread resistance determinant $bla_{CTX\text{-}M\text{-}15}$ is not associated with the gene coding for $bla_{NDM\text{-}1}$ on the same plasmid. This finding is comparable to previous study conducted by Gaëlle Cuzon *et al.*, (2010).

ST 14 type of Kp12 was identified through Multilocus sequence typing (MLST), which is one of the types observed in Indian isolates earlier [Poirel *et al.*, 2011c]. In addition, the same ST14 type was identified in the first NDM-1 producing *K. pneumoniae* isolate from Sweden as well as other isolates with links to contamination in India [Poirel *et al.*, 2011c]. Our data suggest that $bla_{NDM\text{-}1}$ genes benefit transposon location, self-transferable plasmids, by facilitating their rapid spread to *K. pneumoniae* and other bacterial species.

In both strains the surrounding genetic environment was analyzed for the presence of insertion sequences (IS) known to be associated with the $bla_{NDM\text{-}1}$ gene in *Enterobacteriaceae* [Poirel *et al.*, 2011c]. Primers targeting the IS element A*ba*125 identified a remnant of IS*Aba*125 upstream of the $bla_{NDM\text{-}1}$ gene in the Ec15 isolate, whereas an entire IS*Aba*125 element was identified upstream of the $bla_{NDM\text{-}1}$ gene in the Kp12 isolate. A bleomycin resistance gene *ble*MBL was identified downstream of the $bla_{NDM\text{-}1}$ gene in both cases, which encodes a putative bleomycin (an antitumour drug) resistance protein, as reported previously [Poirel *et al.*, 2011c]. These later results indicate that conserved structures surrounding the $bla_{NDM\text{-}1}$ gene are present in NDM-1 producing *Enterobacteriaceae* isolated from north Indian.

6.5 Conclusion

The study provides the information regarding the occurrence of MBL producing clinical isolates in diabetic foot ulcer patients in north India. It also revealed the presence of

bla$_{NDM-1}$ gene in two clinical isolates, *E. cloacae* and *K. pneumoniae*. The present data and earlier reports indicate the need to control the dissemination of carbapenemase producing *Enterobacteriaceae* in the hospital setting, as well as in the community. To control the spread of carbapenem producers, the development of rapid, cheap and easy to handle diagnostic techniques for the identification of carbapenemase producers is required.

Summary of whole study

1. The present work has provided significant insight into the molecular epidemiology of escalating antimicrobial resistance prevalent in ESBL producing *Enterobacteraceae* in India.
2. The study concludes that the independent risk factors for EPK infection are low socioeconomic status and illiteracy of mother.
3. Our findings uphold the increasing role of the bla_{CTX-M} type β-lactamase in antibiotic resistance and stress upon the significance of appropriate empirical treatment for infections.
4. It also confirms that horizontal gene transfer is the most prevalent method of acquiring antimicrobial resistance markers among *Enterobacteraceae* in the community setting.
5. It concludes polyclonal dissemination of ESBL producing strains among clinical isolates of *K. pneumoniae* in a teaching hospital of north India.
6. It provides the information regarding the occurrence of MBL producing clinical isolates in diabetic foot ulcer patients in north India.
7. It also revealed the presence of bla_{NDM-1} gene in two clinical isolates (*E. cloacae* and *K. pneumoniae*) of diabetic foot ulcer patients.
8. The present data and earlier reports indicate the need to control the dissemination of carbapenemase producing *Enterobacteriaceae* in the hospital setting, as well as in the community.
9. In order to prevent the ESBL producing *K. pneumoniae* infection in neonates, non judicial use of antibiotics and common sharing of the unsterilized equipments should be avoided.
10. Moreover, the use of carbapenem, ceftazidime, gatifloxacine, ciprofloxacin and amikacin may enhance the susceptibility of ESBL producing *K. pneumoniae*.
11. To control the spread of carbapenem producers, the development of rapid, cheap and easy to handle diagnostic techniques for the identification of carbapenemase producers is required.
12. Furthermore, efforts are needed to promote the proper use of antibiotics and to discourage their sale over the counter.

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Gene sequences submitted to Gen Bank (Accession numbers)

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